On the Horizon: Targeting Next-Generation Immune Checkpoints for Cancer Treatment

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Abstract

Background: Immune checkpoints are critical regulatory pathways of the immune system which finely tune the response to biological threats. Among them, the CD-28/CTLA-4 and PD-1/PD-L1 axes play a key role in tumour immune escape and are well-established targets of cancer immunotherapy. **Summary:** The clinical experience accumulated to date provides unequivocal evidence that anti-CTLA-4, PD-1, or PD-L1 monoclonal antibodies, used as monotherapy or in combination regimes, are effective in a variety of advanced/metastatic types of cancer, with improved clinical outcomes compared to conventional chemotherapy. However, the therapeutic success is currently restricted to a limited subset of patients and reliable predictive biomarkers are still lacking. **Key Message:** The identification and characterization of

additional co-inhibitory pathways as novel pharmacological targets to improve the clinical response in refractory patients has led to the development of different immune checkpoint inhibitors, the activities of which are currently under investigation. In this review, we discuss recent literature data concerning the mechanisms of action of next-generation monoclonal antibodies targeting LAG-3, TIM-3, and TIGIT co-inhibitory molecules that are being explored in clinical trials, as single agents or in combination with other immune-stimulating agents.

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Introduction

Immune Checkpoint Therapy Overview

The immune checkpoint (ICK) therapy is an emerging approach to oncological treatment, which is increasingly used for a variety of cancers. This strategy relies on the modulation of regulatory pathways that physiologically

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suppress excessive and potentially harmful activation of T lymphocytes during the immune response in order to enhance their natural antitumour activity [1]. Conceptually, the introduction of biological drugs acting on ICKs into clinical practice has represented a key change in the therapeutic approach of cancer, since the target moves from malignant cells to healthy T cells with the ultimate aim of killing tumours by immune-mediated mechanisms. Ipilimumab, a fully human monoclonal antibody (mAb) which targets CTLA-4, a negative regulator of CD28-dependent T-cell responses (Appendix) [2], was the first ICK inhibitor (ICKi) approved by the FDA and EMA in 2011 [3, 4] for patients with metastatic/unresectable melanoma [5, 6]. Ipilimumab also represented the first systemic therapy able to prolong overall survival (OS) in patients with advanced-stage melanoma, more than 30 years following the approval of dacarbazine, a chemotherapeutic agent considered to be the reference drug, although responses were <15% and generally transient [7]. Pooled analysis of survival rates indicated that approximately 20% of all melanoma patients treated with ipilimumab survived for up to 10 years [8], while <2% of dacarbazine-treated patients survived for more than 5 years [9]. However, the majority of patients did not obtain a long-lasting clinical benefit from ipilimumab when used as a single agent. The clinical success of the anti-CTLA-4 mAb has markedly stimulated the development of other ICKi, which in recent years led to the approval of mAbs against the PD-1/PD-L1 axis (Appendix; i.e., the anti-PD-1 mAbs nivolumab, pembrolizumab, and cemiplimab, and the anti-PD-L1 mAbs atezolizumab, durvalumab, and avelumab) [10–12] (Table 1). Even though CTLA-4 and PD-1/PD-L1 seem to cover similar inhibitory effects on T-cell activity (such as the inhibition of T-cell proliferation and survival, reduction in IL-2 and IFN- γ release), they bear distinct profiles of expression and intracellular signalling, and they have non-overlapping biological activity [13] (Appendix). Indeed, for metastatic melanoma, both nivolumab and pembrolizumab showed superior OS and response rates compared to ipilimumab alone [14–17]. Starting from 2014, nivolumab and pembrolizumab were FDA/EMA approved, initially for previously treated, and then for untreated advanced melanoma patients, replacing ipilimumab in the first-line setting. Moreover, combination therapies based on the synergistic blockade of CTLA-4 and PD-1/PD-L1 pathways resulted in enhanced antitumour activity in preclinical mouse models in comparison to either regimen [18]. Indeed, the results of phase III studies showed significantly improved clinical outcomes when nivolumab was

combined with ipilimumab as compared to the single-agent regimen, the 3-year survival rates being 58, 52, and 34% in the nivolumab-plus-ipilimumab, nivolumab, and ipilimumab arms, respectively [14, 15, 19]. Thus, the FDA (in 2015) and EMA (in 2016) approved ipilimumab plus nivolumab as the first ever ICKi combination therapy for the front-line treatment of wild-type BRAFV600 unresectable or metastatic melanoma. Soon after, the indication of nivolumab-ipilimumab combination was extended by FDA to include previously untreated BRAF-mutated melanoma [20]. The introduction of ICKi in clinical practice has also improved survival in patients with other cancer types. Indeed, ICKi are currently approved for a variety of solid tumours, as single agents and in some cases in combination with another ICKi or with chemotherapy (Table 1) [21–24]. Interestingly, pembrolizumab also received FDA approval for the treatment of adult and paediatric patients with unresectable or metastatic solid tumours characterized by microsatellite instability-high (MSI-H) or mismatch repair deficiency (dMMR; Table 1). This represented the first tissue/site agnostic indication of an anticancer drug, based on a tumour biomarker status, rather than on tumour histology. The rationale for using ICKi for MSI/dMMR tumours is based on their elevated mutational burden, which results in the production of a wide repertoire of non-self-antigenic peptides, which in turn can be efficiently targeted by a hyperstimulated immune system [25–29].

ICKi in the Context of the Tumour Microenvironment

A critical aspect in determining the success of ICKi-based therapies regards the increased survival and activation of tumour-infiltrating lymphocytes (TILs) [1]. The T-cell response is an extremely complex process that is enabled through the interaction of both inhibitory and stimulatory signalling pathways coming from cells of different lineages present in the same microenvironment. In cancer progression, tumour microenvironment (TME) is a phenotypic expression of the cross-talk between tumour and healthy host cells, mostly immune cells. A specific TME composition is the result of a vast repertoire of cytokines, soluble-factors, and biological active fragments that orchestrate extracellular matrix (ECM) remodelling and architecture, by affecting cell motility, polarity, and differentiation (in particular epithelial-mesenchymal transition). These factors are either synthesized/secreted by malignant and non-malignant cells or released, upon enzymatic shedding, from structural and non-structural extracellular matrix components (i.e., the tumstatin, canstatin, and arrestin fragments of collagen IV and

Table 1. Approved therapeutic uses of anti-CTLA-4 and anti-PD-1 or anti-PDL-1 mAbs

Target	mAb	FDA approved indication (year of approval)	EMA approved indication (year of approval)
CTLA-4	Ipilimumab	Unresectable or metastatic melanoma (2011)	Unresectable or metastatic melanoma in adults who have received prior therapy (2011)
		Adjuvant treatment for stage III melanoma (2015)	First-line treatment of unresectable or metastatic melanoma in adults (2013)
		Unresectable or metastatic melanoma in paediatric patients (≥ 12 years) (2017)	Unresectable or metastatic melanoma in adults and adolescents (≥ 12 years) (2017)
PD-1	Nivolumab	BRAF V600 wild-type and BRAF V600 mutation positive unresectable or advanced melanoma in previously treated patients (2014)	Unresectable or metastatic melanoma in adults (2015)
		Metastatic squamous NSCLC that progressed during or after platinum-based chemotherapy (2015)	Locally or advanced NSCLC previously treated with chemotherapy (2016)
		Metastatic NSCLC (including non-squamous) that progressed during or after platinum-based chemotherapy (2015)	Advanced renal cell carcinoma after prior therapy in adults (2016)
		Metastatic renal cell carcinoma patients who have received prior antiangiogenic therapy (2015)	Relapsed or refractory classical Hodgkin lymphoma after autologous hematopoietic stem cell transplantation (auto-HSCT) and treatment with brentuximab vedotin (2016)
		Classical Hodgkin lymphoma relapsed or progressed after: (a) auto-HSCT and brentuximab vedotin, (b) 3 or more lines of systemic therapy that includes auto-HSCT (2016)	Locally or metastatic urothelial cancer cannot be surgically removed or previously treated with platinum-based chemotherapy (2017)
		Recurrent or metastatic squamous cell carcinoma of the head and neck with disease progression on or after platinum-based therapy (2016)	Metastatic squamous cell cancer of the head and neck previously treated with platinum-based chemotherapy (2017)
		Locally advanced or metastatic urothelial carcinoma that progressed: (a) during or following platinum-containing chemotherapy; (b) within 12 months of neoadjuvant or adjuvant platinum-containing chemotherapy (2017)	Adjuvant therapy in melanoma with lymph node involvement or metastatic disease after complete resection (2018)
		Adult and paediatric (≥ 12 years) patients with MSI-H or dMMR metastatic colorectal cancer that progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan (2017)	
		Hepatocellular carcinoma previously treated with sorafenib (2017)	
		Adjuvant therapy in melanoma with lymph node involvement or metastatic disease after complete resection (2017)	
CTLA-4 PD-1	Ipilimumab plus nivolumab	Metastatic small cell lung cancer that has progressed after platinum-based chemotherapy (2018)	
		BRAF V600 wild-type unresectable or metastatic melanoma (2015)	Unresectable or metastatic melanoma in adults (2016)
		BRAF V600 wild-type and BRAF V600 mutation-positive unresectable or metastatic melanoma (benefit in low tumour PDL-1 expression) (2016)	Intermediate poor-risk advanced renal cell carcinoma (2018)
PD-1	Pembrolizumab	First-line treatment of intermediate/poor-risk advanced renal cell carcinoma (2018)	
		Adult and paediatric patients (≥ 12 years) with MSI-H or dMMR metastatic colorectal cancer that has progressed after treatment with a fluoropyrimidine, oxaliplatin, and irinotecan (2018)	
		Unresectable or metastatic melanoma patients who are no longer responding to other drugs (2014)	Unresectable or metastatic melanoma (2015)
		Advanced metastatic NSCLC with high PDL-1 expression (tumour proportion score [TPS] $\geq 1\%$) that has progressed after other treatments (2015)	Locally advanced or metastatic non-squamous NSCLC expressing PDL-1 (TPS $\geq 1\%$) after at least one prior chemotherapy regimen (2016)
		First-line treatment of unresectable or metastatic melanoma (2015)	First-line treatment of metastatic NSCLC with no EGFR or ALK-positive mutations expressing PD-L1 (TPS $\geq 50\%$) (2016)
		Recurrent or metastatic head and neck squamous cell carcinoma with disease progression on or after platinum-containing chemotherapy (2016)	Refractory classical Hodgkin lymphoma after failure of auto-HSCT and brentuximab vedotin, or transplant-ineligible and after failure of brentuximab vedotin (2017)
		First-line treatment of metastatic NSCLC with no EGFR or ALK genomic tumour aberrations with high PD-L1 expression (TPS $\geq 50\%$) (2016)	Locally advanced or metastatic urothelial carcinoma not eligible for cisplatin-containing chemotherapy and expressing PD-L1 (combined positive score [CPS] ≥ 10) (2018)
		Adult and paediatric refractory classical Hodgkin lymphoma relapsed after three or more prior lines of therapy (2017)	Locally advanced or metastatic urothelial carcinoma after platinum-containing chemotherapy (2018)
		First-line treatment of metastatic non-squamous NSCLC (in combination with pemetrexed and carboplatin) irrespective of tumour PD-L1 expression status (2017)	First-line treatment of metastatic non-squamous NSCLC with no EGFR or ALK-positive mutations (in combination with pemetrexed and platinum chemotherapy) (2018)
		Locally advanced or metastatic urothelial carcinoma ineligible for cisplatin-containing chemotherapy or progressed during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy (2017)	Recurrent or metastatic head and neck squamous cell carcinoma expressing PD-L1 (TPS $\geq 50\%$), and progressing on or after platinum-containing chemotherapy (2018)
		Adult and paediatric unresectable or metastatic solid tumour with MSI-H or dMMR (2017)	Adjuvant treatment of adults with stage III melanoma and lymph node involvement after complete resection (2018)

Table 1 (continued)

Target	mAb	FDA approved indication (year of approval)	EMA approved indication (year of approval)
		Recurrent locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma expressing PD-L1 (CPS ≥ 1) that has progressed on or after two or more prior lines of therapy including fluoropyrimidine- and platinum-containing chemotherapy and, if appropriate, HER2/neu-targeted therapy (2017)	First-line treatment of metastatic squamous NSCLC (in combination with carboplatin and either paclitaxel or nab-paclitaxel) (2019)
		Recurrent or metastatic cervical cancer with disease progression on or after chemotherapy (PDL-1 expression, CPS ≥ 1) (2018)	
		Adult and paediatric refractory primary mediastinal large B-cell lymphoma, relapsed after two or more prior lines of therapy (2018)	
		First-line treatment of metastatic non-squamous NSCLC with no EGFR or ALK genomic tumour aberration (in combination with pemetrexed and platinum chemotherapy) (2018)	
		First line treatment of metastatic squamous NSCLC (in combination with carboplatin and either paclitaxel or nab-paclitaxel) regardless of tumour PD-L1 expression status (2018)	
		Hepatocellular carcinoma previously treated with sorafenib (2018)	
		Adult and paediatric patients with recurrent locally advanced or metastatic Merkel cell carcinoma (2018)	
		Adjuvant treatment of patients with melanoma with involvement of lymph node(s) following complete resection (2019)	
		First-line treatment of stage III NSCLC not candidate for surgical resection or definitive chemo-radiation, or metastatic NSCLC expressing PD-L1 (CPS $\geq 1\%$), with no EGFR or ALK genomic tumour aberrations (2019)	
		First-line treatment of patients with advanced renal cell carcinoma in combination with the tyrosine kinase inhibitor axitinib (2019)	
PD-1	Cemiplimab	Metastatic or locally advanced cutaneous squamous cell carcinoma not eligible for curative surgery or curative radiation (2018)	
PD-L1	Atezolizumab	Locally advanced or metastatic urothelial carcinoma that has progressed during or following any platinum-containing chemotherapy, or within 12 months of neoadjuvant or adjuvant chemotherapy (2016)	Advanced or metastatic urothelial carcinoma after prior platinum-containing chemotherapy, or cisplatin ineligible with PDL-1 expression covering $\geq 5\%$ tumour area (2017)
		Metastatic NSCLC with disease progression during or following platinum-containing chemotherapy or having progressed on kinase inhibitors if EGFR or ALK mutations are present (2016)	Locally advanced or metastatic NSCLC after prior chemotherapy or that has progressed on kinase inhibitors if EGFR or ALK mutations are present (2018)
		Locally advanced or metastatic urothelial carcinoma not eligible for cisplatin chemotherapy (2017)	
		Urothelial carcinoma not eligible for cisplatin therapy and with PDL-1 expression covering $\geq 5\%$ tumour area (2018)	
		First-line treatment of metastatic non-squamous NSCLC with no EGFR or ALK mutations, in combination with bevacizumab, paclitaxel, and carboplatin (2018)	
		Unresectable locally advanced or metastatic triple-negative breast cancer expressing PD-L1, in combination with nab-paclitaxel (2019)	
		First-line treatment of previously untreated extensive-stage small cell lung cancer in combination with carboplatin and etoposide (2019)	
PD-L1	Avelumab	Metastatic Merkel cell carcinoma in adults and paediatric patients (≥ 12 years) (2017)	Metastatic Merkel cell carcinoma in adults (2017)
		Locally advanced or metastatic urothelial carcinoma that progressed during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant platinum-containing chemotherapy (2017)	
PD-L1	Durvalumab	Locally advanced or metastatic urothelial carcinoma that progressed during or following platinum-containing chemotherapy, or within 12 months of receiving platinum-containing neoadjuvant or adjuvant chemotherapy surgery (2017)	Locally advanced, unresectable NSCLC expressing PD-L1 $\geq 1\%$ that has not progressed following platinum-based chemotherapy and radiation therapy (2018)
		Unresectable stage III NSCLC that has not progressed after treatment with platinum-based chemotherapy and radiation therapy (2018)	

VEGF released from the matrix reservoir) or cell surface proteins (i.e., cytokine/growth factor receptors, adhesion molecules, dystroglycan) [30–34]. In this respect, the pattern of proteolytic enzymes, especially matrix metalloproteinases, released by cancer, immune, and resident cells contribute to the establishment of a tumour-specific TME, as extensively reviewed elsewhere [32, 33, 35].

Thus, TME components tightly affect tumour growth or dissemination and immune responses, and probably also the immunotherapy outcome. For instance, tumour-infiltrating macrophage (TAM) polarization is highly dependent on the type of cytokines produced in the TME, which may determine the generation of pro-inflammatory and tumouricidal M1 or of pro-tumoural M2 TAMs

[36]. The latter, besides directly promoting tumour growth and metastasis through the secretion of matrix metalloproteinases, growth factors, and angiogenic factors, efficiently suppress immune effector functions by producing cytokines and enzymes, which directly or indirectly suppress effector cells [37]. Interestingly, the cross-talk between T cells and TAMs [38] may result in a dual opposite effect: (a) increased recruitment of cytotoxic T cells sustained by M1 TAMs cytokines/chemokines; (b) blockade of cytotoxic T-cell activity by M2 TAMs. Since the M2-induced negative feedback pathway on T cells can hamper the efficacy of immunotherapies, it has been suggested that inhibition of M2 TAMs, by targeting specific receptors expressed in these cells (e.g., VEGFR-1), might improve ICKi efficacy [39–42]. Therefore, the extreme heterogeneity of TME composition makes it hard to decipher how a given TME drives the immune response to cancer and, consequently, how it may affect the overall efficacy of ICKi [43].

The Future of ICK Therapy

Despite the success of the ICK blockade, many questions remain unsolved. This might account for the evidence that clinical benefit is limited to a subset of patients for each cancer type and, additionally, responders often develop resistance to treatment [44]. Due to the dynamic nature and complexity of the immune response, there is increasing interest in understanding the molecular mechanisms of “novel” immunomodulatory pathways and the molecular bases of the ICK interplay. The combination of ICKi either with ICKi targeting different immune regulatory pathways or with conventional anticancer therapy (chemotherapy and radiotherapy) has emerged as a promising approach to improving clinical benefit. Moreover, immune-directed strategies hitting different TME components that regulate the multifaceted aspects of cancer interaction are currently under investigation [45–47]. The main “novel” ICKs that are involved at different steps in immune antitumour responses include either positive or negative modulators. Negative modulators are LAG-3, TIM-3, TIGIT, VISTA, B7-H3, and BTLA, which assist the CTLA-4 and PD-1/PD-L1 pathways in turning off the physiological immune response, albeit with some variability which has emerged depending on the model [22, 44]. Conversely, immune activators include, among others, ICOS, a member of the CD28-superfamily expressed in activated T cells [48], and CD40, a member of the TNF receptor superfamily, which is expressed in a number of cells of the innate and adaptive immunity as well as in B lymphocytes [49]. Glucocorticoid-induced tumour ne-

crosis factor receptor-related (GITR) is another co-stimulatory molecule of clinical interest. In fact, anti-GITR agonistic mAbs, which induce activation of CD8+ effector function and inhibition of tumour-infiltrating T-regulatory cells (Tregs), are investigated in phase I/II trials [50]. Actually, TME Tregs play a pivotal role in cancer cell escape from immune surveillance and the expression of co-inhibitory molecules, such as CTLA-4, PD-1, LAG-3, TIM-3, and TIGIT (see below), on their surface is critical for their suppressive functions [51]. Other T-cell co-stimulatory receptors that have been targeted by agonist mAbs in clinical development include CD27, OX-40, 4-1BB, and TWEAK [52–54].

Here, we review the biological functions and molecular features of LAG-3, TIM-3, and TIGIT, three well-studied novel ICKs that strictly co-operate with CD28/CTLA-4 and PD-1/PD-L1 axes to modulate the cancer immune response. An update on the clinical development of agents targeting these ICKs (e.g., mAbs including bispecific antibodies, recombinant fusion proteins) is also presented (Table 2).

Lymphocyte Activation Gene-3 (LAG-3, CD223)

Structural Molecular Features and Signal Transduction of LAG-3

Lymphocyte activation gene-3 (LAG-3 or CD223) is a promising ICK, which physiologically suppresses T-cell activation and cytokine secretion. LAG-3 is a single transmembrane protein with three Ig extracellular domains that is expressed on activated CD4⁺ and CD8⁺ T cells, Tregs, B cells, natural killer (NK), and dendritic cells (DC) [55–61]. Even though the amino acid sequence homology is less than 20%, LAG-3 shares high structural homology with CD4 and both genes are located proximally on human chromosome 12 (12p13) [61, 62]. Like CD4, LAG-3 binds to major histocompatibility complex MHC class II (MHC-II) molecules (Fig. 1), although its K_d (6×10^{-8} M) is several orders of magnitude higher than that of CD4 (10^{-4} M) [63, 64]. Surprisingly, despite the high affinity, only a handful of residues located on the D1 loop are involved in MHC-II and LAG-3 binding, in contrast to the extensive molecular interaction between MHC-II and CD4 [65, 66].

Several studies initially performed in cellular in vitro models and then confirmed in murine models revealed that the interaction between LAG-3 and MHC-II hinders the MHC-II binding to the T-cell receptor (TCR) and CD4, thus directly suppressing the TCR signalling and

Table 2. Clinical trials with next-generation ICKi

Target	Drug	Combined agent	NCT identifier	Phase	Status	Setting
LAG-3	Eftilagimod alpha (IMP321) (soluble LAG-3 Ig)	–	NCT00732082 [98]	I	Terminated	Unresectable pancreatic cancer receiving first-line gemcitabine
		–	NCT00349934 [100]	I	Completed	Metastatic breast carcinoma
		–	NCT00351949 [97]	I	Completed	Renal cell carcinoma
		Melan-A VLP vaccine	NCT00324623 [99]	I	Completed	Melanoma
		–	NCT03252938	I	Recruiting	Solid tumours
		Pembrolizumab	NCT02676869	I	Active, not recruiting	Unresectable or metastatic melanoma
		Paclitaxel	NCT02614833	I/II	Recruiting	Hormone receptor-positive metastatic breast cancer
		Pembrolizumab	NCT03625323	II	Recruiting	NSCLC and head and neck carcinoma
	Relatlimab	Nivolumab and urelumab (anti-CD137)	NCT02658981	I	Recruiting	Recurrent glioblastoma
		Nivolumab	NCT02966548	I	Recruiting	Advanced solid tumours
		Nivolumab, carboplatin, paclitaxel, radiotherapy	NCT03044613	I	Recruiting	Neoadjuvant treatment of stage II/III oesophageal/gastro-oesophageal junction cancer
		Nivolumab	NCT03493932	I	Recruiting	Recurrent glioblastoma
		Nivolumab	NCT01968109 [102, 103]	I/II	Recruiting	Unresectable and metastatic solid tumours
		Nivolumab	NCT02061761	I/II	Recruiting	Relapsed hematologic malignancies
		Nivolumab, ipilimumab, daratumumab (anti-CD38)	NCT02488759	I/II	Recruiting	Virus-associated tumours
		Nivolumab and BMS-986205 (IDO1 inhibitor) or nivolumab and ipilimumab	NCT03459222	I/II	Recruiting	Advanced/metastatic solid tumours
		Nivolumab	NCT03610711	I/II	Not yet recruiting	Recurrent or metastatic oesophageal-gastric cancer
		Ipilimumab, nivolumab, conventional surgery	NCT02519322	II	Recruiting	Unresectable stage IIIB-IV melanoma
		Nivolumab	NCT03607890	II	Recruiting	MSI-H solid tumours refractory to prior PD-L1 therapy
		Nivolumab	NCT03623854	II	Not yet recruiting	Advanced chordoma
		Nivolumab	NCT03642067	II	Recruiting	Metastatic or locally advanced micro-satellite stable (MSS) colorectal cancer
		Nivolumab	NCT03743766	II	Recruiting	Unresectable or metastatic melanoma patients who have not received prior treatment with immunotherapy
		Nivolumab	NCT 03470922	II/III	Recruiting	Unresectable or metastatic melanoma patients who have not received prior treatment with immunotherapy
	LAG525 (mAb)	Spartalizumab (anti-PD-1), NIR178 (adenosine A2A receptor antagonist), capmatinib (c-met inhibitor), MCS110 (anti-M-CSF), canakinumab (anti-IL-1 β)	NCT03742349	I	Recruiting	Triple negative breast cancer
		Spartalizumab	NCT02460224	I/II	Active not recruiting	Advanced tumours
		Spartalizumab	NCT03365791	II	Active not recruiting	Advanced solid and haematological malignancies
Sym022 (mAb)	–	–	NCT03489369	I	Recruiting	Advanced/unresectable or metastatic solid tumour or lymphomas refractory to available therapy
		Sym021 (anti-PD-1) and Sym023 (anti-TIM3)	NCT03311412	I	Recruiting	Advanced solid tumours or lymphomas
INCAGN02385 (mAb)	–	–	NCT03538028	I	Recruiting	Advanced malignancies
REGN3767 (mAb)	REGN2810 (anti-PD-1)	–	NCT03005782	I	Recruiting	Advanced malignancies including lymphoma
TSR-033 (mAb)	Anti-PD-1	–	NCT03250832	I	Recruiting	Advanced solid tumours

Table 2 (continued)

Target	Drug	Combined agent	NCT identifier	Phase	Status	Setting
	MGD013 (PD-1/LAG-3 bispecific mAb)	–	NCT03219268	I	Recruiting	Advanced solid tumours and haematological malignancies
	FS118 (LAG-3/PD-L1 bispecific mAb)	–	NCT03440437	I	Recruiting	Tumours that have progressed on or after prior PD-1/PD-L1-containing therapy
	BMS-986213 (fixed dose combination of relatlimab and nivolumab)	Chemotherapy	NCT03662659	II	Recruiting	Gastric or gastroesophageal junction adenocarcinoma
TIM-3	Sym023 (mAb)	–	NCT03489343	I	Recruiting	Advanced/unresectable or metastatic solid tumour or lymphomas that are refractory to available therapy or for which no standard therapy is available
		Sym021 (anti-PD-1) and/or Sym022	NCT03311412	I	Recruiting	Advanced/unresectable or metastatic solid tumour malignancies or lymphomas that are refractory to available therapy or for which no standard therapy is available
	TSR-022 (mAb)	An anti-PD-1 mAb	NCT02817633	I	Recruiting	Advanced solid tumours
		Niraparib (PARP inhibitor); TSR-042 (anti-PD-1); carboplatin; paclitaxel; bevacizumab; pemetrexed	NCT03307785	I	Recruiting	Metastatic and advanced solid tumours; metastatic and stage IIIB NSCLC
		TSR-042 (anti-PD-1)	NCT03680508	II	Recruiting	Liver cancer
	INCAGN02390 (mAb)	–	NCT03652077	I	Recruiting	Advanced malignancies
	LY3415244 (anti-PD-L1/TIM-3 bispecific Ab)	–	NCT03752177	I	Recruiting	Advanced solid tumours
	LY3321367 (mAb)	LY3300054 (anti-PD-1)	NCT03099109	I	Recruiting	Advanced relapsed/refractory solid tumours
	MBG453 (mAb)	Spartalizumab	NCT02608268	I	Recruiting	Advanced solid tumours
		Spartalizumab and decitabine	NCT03066648	I	Recruiting	AML and high-risk MDS patients
TIGIT	BGB-A425 (mAb)	Tislelizumab (anti-PD-1)	NCT03744468	I	Recruiting	Advanced solid tumours
	RO7121661 (anti-PD-1/TIM-3 bispecific mAb)	–	NCT03708328	I	Recruiting	Advanced and/or metastatic solid tumours
	OMP-31M32 (mAb)	Nivolumab	NCT03119428	I	Active not recruiting	Locally advanced or metastatic solid tumours
	AB154 (mAb)	AB122 (anti-PD-1)	NCT03628677	I	Recruiting	Advanced malignancies
	MTIG7192A (mAb)	Atezolizumab	NCT03563716	II	Recruiting	Chemotherapy-naïve patients with locally advanced or metastatic NSCLC

preventing activation of immune responses [63, 67–69]. It has been further shown that LAG-3 spatially associates with the TCR/CD3 complex clustered in raft microdomains [66, 70, 71]. A detailed understanding of the LAG-3 intracellular signalling pathway is still lacking and this is likely related to the fact that the LAG-3 cytoplasmic tail lacks canonical inhibitory motives (such as immunoreceptor tyrosine-based inhibitory motif [ITIM] and immunoglobulin tyrosine domain [ITT]) present in other immune receptors. In fact, from a structural point of view, the intracellular region consists of three domains: (i) a serine-phosphorylation site, (ii) a unique KIEELE motif, and (iii) a glutamic acid-proline (EP) repeat [67, 69, 72–74]. The KIEELE motif transmits the inhibitory signal and plays a crucial role in LAG-3 negative regulatory function (Fig. 1), since LAG-3 molecules lacking this domain cannot negatively modulate T-cell activity [74].

LAG-3 in Immune Tolerance to Tumours

The LAG-3 pathway directly regulates the function of T cells (Fig. 1). In CD4⁺ lymphocytes, LAG-3 signalling prevents the entry of T cells in the S phase of the cell cycle, thus arresting CD4⁺ clone expansion and suppressing cytokine secretion [60, 67]. However, LAG-3 is also a negative regulator of CD8⁺ T cells, where it is upregulated following antigen stimulation [68, 69, 75]. Interestingly, tumour-infiltrating CD8⁺ cells overexpress LAG-3 in various cancer types (i.e., ovarian cancer, renal cancer, and hepatocarcinoma) [76–79], and the LAG-3 blockade increases CD8⁺ T-cell effector function independently from CD4⁺ T-cell activity [80]. Recently, LAG-3 has been reported to interact with two additional ligands, galectin-3 (Gal-3), a lectin that is secreted by many cells in TME, and liver sinusoidal endothelial cell lectin (LSECtin), a cell surface lectin expressed by cancer cells (Fig. 1) [66]. The

Fig. 1. LAG-3 signalling in TME. LAG-3 is expressed mainly on T and NK cells and binds to MHCII (expressed on APC), Gal-3 (released in TME), and LSECtin (expressed on tumour cells). The LAG-3 cytoplasmic tail contains a non-canonical inhibitor motif KIEELE that mediates an unknown downstream molecular cascade, providing LAG3 with a negative regulatory function (see text for further details).

LAG-3 interaction with these two alternative ligands promotes tumour growth by inhibiting antitumour CD8⁺-dependent responses (Fig. 1) [81–83]. LAG-3 expression is also increased in many tumour-infiltrating Tregs, such as in head and neck squamous small-cell carcinoma and non-small cell lung cancer (NSCLC) [84, 85], and significantly contributes to the suppressive functions of these cells [59, 80]. In fact, the interaction between the MHC-II expressed on antigen-presenting cells (APCs) with LAG-3 on Tregs maximizes the production of immunosuppressive cytokines such as IL-10 and TGF- β , thus inhibiting cytotoxic T-cell activity [59, 60, 86, 87]. Moreover, this interaction contributes to suppressing DC activation, through the immunoreceptor tyrosine-based activation motif (ITAM)-mediated inhibitory pathway, and to regulating DC homeostasis [58, 88, 89]. Therefore, LAG-3 inhibition might block this suppressive pathway and, consequently, stimulate the antitumour activity of CD8⁺ TILs. LAG-3 exhibits synergistic immunosuppressive ef-

fects with other ICKs, mainly with PD-1/PD-L1 [90]. PD-1 and LAG-3, which are co-expressed on the surface of CD4⁺ and CD8⁺ cells, particularly in TILs, co-operate in regulating T-cell function to promote tumour immune escape [76, 79, 91–94]. The synergistic effect of LAG-3 and PD-1 co-inhibition in enhancing antitumour immunity has been demonstrated using LAG-3 and PD-1 double-KO mice, in which implanted tumours were rejected, whereas in PD-1 KO mice the same tumours only showed a delayed growth [13, 69, 95]. Likewise, a combined blockade of both ICKs by using anti-PD-1 and anti-LAG-3 mAbs potentiated the immune response against tumours [13, 69, 91, 95, 96]. Therefore, the available data suggest that therapeutic strategies combining LAG-3 and PD-1 blockers might improve the clinical benefit in different tumour types. Accordingly, a number of phase I/II clinical trials are being carried out to evaluate drug combinations which simultaneously target these two ICKs (Table 2) [69].

LAG-3 Targeting in Immunotherapy

Currently, two main approaches directed against LAG-3 have been developed and are being investigated in different phases of clinical trials: (i) a LAG-3-Ig fusion protein (IMP321 or efitlagimod alpha) and (ii) mAbs which target LAG-3 (BMS-986016 or relatlimab; LAG525 or IMP701; REGN3767; TSR-033; Table 2). Recently, three phase I clinical trials evaluating IMP321 for renal carcinoma, pancreatic adenocarcinoma, and melanoma have been completed with promising results in terms of proliferation and activation of TILs at the tumour site, but not in terms of OS of treated patients [97–99]. The results of another completed phase I clinical trial in metastatic breast carcinoma, reporting a 50% objective response rate with IMP321 plus paclitaxel versus 25% with paclitaxel alone [100], have prompted a phase IIb clinical trial that is currently recruiting patients with hormone receptor-positive metastatic breast cancer (NCT02614833). A number of phase I and II clinical trials are recruiting patients to evaluate anti-LAG-3 mAbs as single agents or in combination with chemotherapy, targeted agents, or various anti-PD-1 mAbs (www.clinicaltrials.gov) [69, 90] (Table 2). Notably, interim analysis of clinical studies evaluating the first developed anti-LAG-3 antibody (BMS-986016 or relatlimab) with nivolumab in unresectable/metastatic melanoma patients showed promising activity in terms of objective response and disease control rates in patients who were not responsive to previous anti-PD-1 monotherapy. In addition, the response rate was higher in LAG-3-overexpressing tumours ($\geq 1\%$) and the safety profile was similar to that of nivolumab monotherapy [101–104]. A phase II trial is currently evaluating the efficacy and safety of relatlimab in combination with nivolumab versus nivolumab alone in previously untreated metastatic/unresectable melanoma. Interestingly, an innovative approach targeting LAG-3 and PD-1 or PD-L1 with bispecific mAbs (MGD013 and FS118, respectively) is also undergoing phase I clinical trials (Table 2; www.clinicaltrials.gov) [69].

TIM-3

Molecular Features, Ligands, and Functions of TIM-3

TIM-3 (T-cell immunoglobulin- and mucin domain-containing molecule 3) is a type I transmembrane protein, identified on the surface of CD4⁺ and CD8⁺ cells, Tregs, DCs, macrophages, NK cells, B and mast cells. TIM-3 was originally discovered in murine models of autoimmune encephalomyelitis as a negative regulator of

the T helper type-1 (Th1) response and Th1-related cytokine expression, such as TNF and INF- γ [105–107]. Alteration of the TIM-3 expression profile as well as the TIM-3 blockade result in the exacerbation of autoimmune diseases such as multiple sclerosis, type I diabetes, and rheumatoid arthritis [108–114]. In recent years, corroborating evidence has clarified that TIM-3 further inhibits antitumour immunity, mainly by stimulating T-cell exhaustion and a number of preclinical studies support the hypothesis that modulation of the TIM-3 pathway might improve cancer immunotherapy outcome [115–121]. However, controversial tasks regarding the biological role of the TIM-3 signalling pathway still exist, since some studies suggest a stimulatory role of TIM-3 on T-cell function [114, 122].

From a structural point of view, TIM-3 is composed of: (i) an extracellular N-terminal IgV domain followed by a membrane-proximal mucin-like domain that contains O-linked glycosylation sites, and a domain with N-linked glycosylation sites, (ii) a transmembrane domain, and (iii) a C-terminal cytoplasmic tail [114].

The first discovered extracellular ligand of TIM-3 was galectin-9 (gal-9), a β -galactoside lectin protein that specifically recognizes the structure of N-linked sugar chains in the Tim-3 IgV domain. This ligand is widely expressed in human tumours such as haematological malignancies (Fig. 2). TIM-3/gal-9 interaction has been reported to alter calcium flux and to induce cell death in Th1 cells, thus suppressing their function [109]. Moreover, in colon cancer, tumour-derived gal-9 induces apoptosis of tumour-infiltrating CD8⁺ TILs [123]. However, TIM-3/gal-9 interaction possibly further modulates intracellular signalling by stimulating cytokine production and secretion [124, 125]. Thus, the biological role of this interaction in T-cell function remains unclear [114].

Phosphatidylserine (PtdSer) is a non-protein ligand of TIM-3 which is exposed on apoptotic cell membranes, and binds the IgV domain of TIM-3 on macrophage surfaces, contributing to the clearance of apoptotic bodies [126, 127] (Fig. 2). Another TIM-3 ligand primarily involved in the innate immune response is high-mobility group box 1 (HMGB-1), which is secreted by immune cells as an inflammatory mediator. In TME, HMGB-1 binds to the nucleic acids released from dying cells and mediates their internalization into DC endosome vesicles after binding to receptors for advanced glycation end products (RAGE) and Toll-like receptors, thus triggering natural immunity [128, 129] (Fig. 2). Remarkably, tumour-infiltrating DCs overexpress TIM-3, and this receptor competes with nucleic acids for the binding to

Fig. 2. TIM-3 signalling in TME. TIM-3 receptor is mainly expressed in T, NK, and APC cells and binds to four ligands in TME: two soluble molecules, Gal-9 (which specifically recognizes the structure of N-linked sugar chains in the TIM-3 extracellular domain), and HMGB1, and two cell surface molecules, PtdSer, and Ceacam-1. In the absence of ligand, the cytoplasmic tail of TIM-3 binds to Bat3, preventing downstream signalling. Ligand/TIM-3 interaction induces the dissociation of Bat3 and the phosphorylation of tyrosine residues by Fyn kinase. This event leads to the recruitment of molecular intracellular adaptors (such as p85) and triggers a downstream inhibitory intracellular signalling (see text for further details).

HMGB-1. TIM-3/HMGB-1 interaction inhibits the recruitment of nucleic acids within endosomes, dampening the tumour-derived nucleic acid activation of innate immune response [129, 130]. The most recently identified TIM-3 ligand is carcinoembryonic antigen cell adhesion molecule 1 (Ceacam-1), a surface molecule that is co-expressed with TIM-3 in CD4⁺ T cells following tolerance induction, and in exhausted CD8⁺ TILs. Ceacam-1 facilitates the maturation and stability of TIM-3 through a *cis*-heterodimeric interaction, and stimulates, through *cis* and/or *trans* mechanisms, T-cell immune tolerance (Fig. 2) [131]. Even though all the above-described mechanisms may contribute to explaining the immunosuppressive properties of TIM-3, how the binding to its ligands and the cross-talk between the activated pathways exactly impact on the immune response is largely unexplored. Although research into antibodies targeting TIM-3 is ongoing in clinical trials, their ability to inhibit the interaction of TIM-3 with its different ligands is not always documented [132].

TIM-3 Intracellular Signalling

A peculiar structural feature of TIM-3 is that its cytoplasmic tail lacks any known canonical inhibitory sequence, such as ITIM and ITT. Instead, it displays a con-

served tyrosine-based motif that mediates the intracellular signalling pathway (Fig. 2) [133]. Several unresolved issues still exist regarding the TCR signalling and the metabolic pathways actually regulated by TIM-3, as emphasized by its pleiotropic activities under the different pathophysiological conditions cited above. In fact, the phosphorylation of 265 and 272 tyrosine residues by different kinases (e.g., Fyn) promotes downstream recruitment of one or more SH2 domain-containing proteins (including the p85 adaptor of PI3K and PLC- γ), leading to the inhibition of TCR-mediated NFAT/AP-1/NF- κ B activation (Fig. 2) [114, 122, 134]. Conversely, transient TIM-3 expression was reported to increase TCR signalling, even after deleting most of its ectodomain [122]. In the absence of extracellular ligands, the molecular adaptor Bat3 binds to the TIM-3 intracellular tail, preventing TIM-3-mediated cell death or exhaustion of T cells. Ligand binding (such as gal-9 and Ceacam-1) results in the phosphorylation of 265 and 272 tyrosine residues, and in the release of Bat3, thus promoting TIM-3-mediated inhibitory functions. This allows the binding of SH2 domain-containing proteins and subsequent regulation of TCR signalling. Since Fyn and Bat3 compete for the binding to the TIM-3 cytoplasmic tail, the molecular switch between TIM-3/Bat3 and TIM-3/Fyn complexes might

be a major event in determining whether TIM-3 positively or negatively regulate TCR activation based on the features of the specific TME (Fig. 2) [131, 132, 135]. Remarkably, even though Ceacam-1 and gal-9 bind to two different sites on the TIM-3 extracellular domain, their interaction with the receptor mediates similar downstream intracellular events, suggesting that the two signalling pathways could have synergistic effects on TIM-3 function [114].

Targeting TIM-3 for Cancer Immunotherapy

As mentioned above, despite some controversies, there is a general agreement in considering TIM-3 to be a negative regulator of antitumour responses by modulating CD8⁺ T-cell exhaustion and the Th1 response. In different tumours (e.g., melanoma, NSCLC, hepatocellular carcinoma, and cervical cancer), TIM-3 expression is upregulated in CD4⁺ and CD8⁺ TILs [136–143], and its expression associates with exhaustion of CD8⁺ T cells in metastatic melanoma and NSCLC [115, 144–145]. Interestingly, TIM-3 upregulation is usually associated with that of PD-1 and TIM-3⁺/PD-1⁺ double-positive TILs exhibit the most severe exhausted phenotype, with marked inhibition of T-cell proliferation and reduction in IL-2, TNF, and IFN- γ release [114, 116]. This evidence provides a rationale for the co-administration of anti-PD-1 and anti-TIM-3 mAbs to achieve a more effective control of tumour growth [114, 116]. A consistent amount of TIM-3⁺/CD4⁺ TILs expresses FoxP3 in a variety of tumours (e.g., hepatocellular, cervical, colorectal, and ovarian cancers), suggesting a role for TIM-3 in the regulation of Treg functions in TME [138, 146]. In NSCLC, the presence of TIM-3⁺ Tregs correlates with unfavourable clinical-pathological parameters [145]. Additionally, tumour-derived TIM-3⁺/FoxP3⁺ Treg populations reveal a more suppressive capacity compared to TIM-3⁻/FoxP3⁺ cells, inhibiting CD8⁺ TIL proliferation and favouring T-cell exhaustion. Accordingly, depletion of TIM-3⁺ Tregs is associated with an increase in CD8⁺ T cells that no longer exhibit an exhausted phenotype [138, 145, 146]. TIM-3⁺ Tregs also co-express high levels of PD-1, supporting a functional relationship between PD-1 and the TIM-3 pathway in T cells with an exhausted phenotype [114, 132, 138, 145]. Accumulating evidence also reveals that tumour cells themselves can express TIM-3 and this phenotype correlates with a poor prognosis [136, 147–153]. However, even though TIM-3 expression on the tumour cell surface seems to mediate immune escape through different mechanisms, ranging from the promotion of tumour cell migration to inhibi-

tion of CD4⁺ functionality [149, 150, 153], the molecular basis of TIM-3-mediated tumour/immune cell cross-talk is far from being completely understood.

Overall, the above-mentioned studies support the rationale for combining agents targeting TIM-3 with anti PD-1 mAbs [116]. In particular, in murine models, a TIM-3 and PD-1 dual blockade has been reported to overcome resistance to PD-1 monotherapy [154]. In this context, phase I clinical trials are evaluating the safety profile of anti-TIM-3 mAbs (i.e., Sym023; TSR-022; INCAGN02390; LY3321367; MBG453; BGB-A425), alone or in combination with anti-PD-1 mAbs, targeted agents or chemotherapy, and of anti-TIM-3/PD-L1 and anti-TIM-3/PD-1 bispecific antibodies (i.e., LY3415244 and RO7121661, respectively) for the treatment of different solid tumours (Table 2; www.clinicaltrials.gov).

TIGIT

Structural Characteristics and Signal Transduction of TIGIT

TIGIT (T-cell immunoreceptor with immunoglobulin and ITIM domains) is a member of the poliovirus receptor (PVR)-nectin family that inhibits T and NK cell activity and whose expression is tightly restricted to lymphocytes [155–157]. The high expression in many cells involved in cancer immunosurveillance (Tregs, CD4⁺, CD8⁺, and NK cells) makes TIGIT a promising target for cancer immunotherapy [156, 158–162]. Importantly, TIGIT expression defines Foxp3⁺ Tregs, whereas in CD8⁺ lymphocytes it marks dysfunctional cells that co-express LAG-3, TIM-3, and PD-1 (see below) [157]. The TIGIT receptor is composed of: (i) an extracellular IgV domain, (ii) a transmembrane region, and (iii) a cytoplasmic tail that contains classical inhibitory motifs, ITIM, and ITT (unlike TIM-3 and LAG-3) [155, 163] (Fig. 3). The two main TIGIT ligands, CD155 (PVR) and CD112 (PVRL2), which are expressed on APC membranes, T and tumour cells [164–166], also bind to immune-activating receptor CD226 (DNAM-1). Contrary to TIGIT, CD226 enhances cytotoxicity of T lymphocytes and NK cells toward tumour cells [157]. The CD155-CD112/CD226/TIGIT axis shares molecular similarities with the B7/CD28/CTLA-4 pathway. In fact, TIGIT (like CTLA-4) binds to its ligands with higher affinity than CD226 and transmits an inhibitory downstream intracellular signalling that dampens the positive signal triggered by the CD155-CD112/CD226 interaction [162, 165]. In NK cells, where TIGIT signal-

ling has been extensively studied, receptor activation induces a marked reduction of IFN- γ release along with a significant decrease in cytotoxic activity [165, 167–169]. TIGIT/ligand interaction induces the phosphorylation of tyrosine residues in the ITT (Y225) and ITIM (Y231) cytoplasmic domains by Fyn and Lck kinases, and recruitment of phosphatase SHIP1 and SHIP 2 (through the adapter Grb2 and β -arrestin 2). This event prevents the activation of PI3K and NF- κ B cascades, inhibiting NK effector function (Fig. 3) [162, 165, 167–169].

TIGIT Role in Immune Biology of Cancer

Apart from the well-characterized role in mediating NK function, in the context of TME, TIGIT/CD155 interaction also modulates the activity of DCs by stimulating the secretion of IL-10 and inhibiting that of IL-12, concurring in the acquisition of a tolerogenic phenotype [155]. These phenomena bring about the inhibition of IFN- γ release and T-cell proliferation [155, 166]. Therefore, TIGIT switches off the activity of key immune cells through a modification of the cytokine balance [160]. TIGIT is expressed in Foxp3⁺ Tregs, and it is fundamental in the maturation process of naïve T cells toward a Treg phenotype [157, 161]. In fact, TIGIT⁺ Tregs display higher immunosuppressive properties compared to TIGIT[−] Tregs [161, 170, 171]. Importantly, TIGIT⁺/Foxp3⁺ cells suppress pro-inflammatory Th1 and Th17 responses, but spare Th2 response through a fibrinogen-like 2-dependent mechanism and shift the cytokine production toward IL-10 [161, 172]. However, the role of the TIGIT pathway in mediating Treg response in the regulation of tumour growth is unclear, even though in murine models, TIGIT seems to play a greater role on Tregs than on CD8⁺ T cells in dampening the antitumour immune response [171]. Indeed, it has been proposed that TIGIT⁺ Tregs are involved in the initial phase of tumour development, but, once the tumour is grown, TIGIT appears to be more relevant in the regulation of CD8⁺ response [162, 171]. In this context, it is worth mentioning that TIGIT is expressed at high levels on TILs in a broad range of tumours, and that, in the TME, TIGIT expression correlates with a marked CD8⁺ dysfunctional phenotype [156, 166, 171, 173]. In fact, the TIGIT pathway directly inhibits T-cell activation and effector functions in a cell-specific manner by targeting TCR signalling (independently of APC-mediated mechanisms), which leads to a reduced secretion of pro-inflammatory cytokines and a reduced degranulation, supported by an increase in the secretion of the immunosuppressive cytokine IL-10 [159, 161].

Fig. 3. TIGIT signalling in TME. TIGIT expression is restricted to lymphocytes. It binds two main ligands, CD155 and CD112, expressed in APC and tumour cells, and contains two classical inhibitory motives, ITIM and ITT in the cytoplasmic tail. Ligand/TIGIT interaction induces the phosphorylation of tyrosine residues in the ITT and ITIM regions by Fyn and Lck kinases, and the recruitment of SHIP1 and SHIP2 proteins. This event leads to NK and T-cell suppression (see text for further details).

Targeting TIGIT in Cancer Immunotherapy

As discussed above, TIGIT modulates antitumour response in multiple steps, since it is involved in the inhibition of NK effector functions, suppression of DC costimulatory properties, modulation of Treg response, and the inhibition of CD8⁺ cytotoxicity [162]. Importantly, CD8⁺ TILs with the most exhausted phenotype co-express PD-1, TIM-3, and TIGIT [156, 166, 171, 173, 174], thus the simultaneous blockade of PD-1/PD-L1 (and/or TIM-3) and TIGIT might be a useful strategy to restore the functionality of CD8⁺ TILs [156, 166, 173]. Indeed, administration of mAbs against TIGIT and PD-1 in the CT26 murine colon carcinoma model induced tumour rejection and reverted CD8⁺ exhaustion [156]. Accordingly, in CD8⁺ TILs from melanoma patients, a combined blockade of TIGIT and PD-1 increased proliferation, cytokine production, and degranulation process [173]. Moreover, TIGIT knockdown restored cytokine production in CD8⁺ TILs from acute myeloid leukaemia patients

[175]. An intriguing open question concerning the synergistic co-blockade of TIGIT and of PD-1/PD-L1 axes regards whether or not immunostimulation mainly derives from the functional restoration of exhausted T cells within the tumour and/or from an increase of CD8⁺ cell recruitment from the lymph nodes to the tumour site [156, 162, 176, 177]. Consistently with these findings, a number of phase I clinical trials with mAbs directed toward TIGIT, alone or in combination with anti PD-1 or PD-L1 mAbs, are currently ongoing in patients with solid tumours (Table 2; www.clinicaltrials.gov).

Conclusion

The successful application of the anti-CTLA-4 and anti-PD-1/PD-L1 mAbs has paved the way to a new era of anticancer therapy changing the therapeutic scenario in a number of tumour settings. However, clinical benefit is still limited to a subset of patients, which is likely due to the heterogeneity of the cancer immunogenic profile during growth and dissemination, and acquisition of resistance mechanisms. In fact, TME undergoes continuous remodelling during tumour progression and TME features vary in primary and metastatic sites. Therefore, a major challenge is to assess the precise molecular features of a given TME and the dynamic interplay between immune and cancer cells in the context of TME. However, with the exception of PD-L1 for certain tumour types, in most cases reliable tumour- or TME-associated biomarkers to stratify patients into responder and non-responder categories are not yet available. The clinical experience accumulated to date with the co-administration of nivolumab and ipilimumab has strengthened the evidence that the combination of drugs targeting different ICK regulatory pathways is a feasible approach to improve treatment efficacy and potentially delay or prevent tumour resistance. Moreover, in order to further increase the proportion of patients that might obtain durable responses, a better understanding of the immune regulatory networks of stimulatory and inhibitory pathways in the context of TME is mandatory.

Over the last decade, the landscape of inhibitory pathways has expanded to include LAG-3, TIM-3, and TIGIT, which display functional redundancy with CTLA-4 and PD-1/PD-L1 axes, but also unique molecular signalling associated with specific regulatory roles. A recently proposed model [44, 166] envisages that immune surveillance fulfilment is guaranteed by the hierarchical organization of co-inhibitory pathways, in which CTLA-4 and

PD-1/PD-L1 axes dominate the scenario, whereas LAG-3, TIM-3, and TIGIT represent the second line of action. However, some aspects of functional redundancy of these different pathways suggest that they might co-operate to maintain immune homeostasis. Therefore, it is possible that the “spatial” and “temporal” differences of the first- (CTLA-4 and PD-1/PD-L1) and the second-line (LAG-3, TIM-3, TIGIT) pathways are not so marked. Therapies targeting LAG-3, TIM-3, and TIGIT, either as monotherapy or in combination with other ICKi, are undergoing clinical development. In this regard, a comprehensive understanding of the role played by these (and other) “new generation” ICKs in the immune response against different cancer types might offer unprecedented therapeutic opportunities, particularly in the context of combinatorial strategies with “first-generation” immunotherapies and/or other targeted agents.

Appendix

The cytotoxic T-lymphocyte antigen 4 (CTLA-4) acts as a negative regulator of the co-stimulatory signal that is required for T-cell activation [2, 47, 178]. According to the double-signal model, two simultaneous events are required for primary T-cell activation. The first signal is represented by the binding of TCR to an MHC molecule of an activated APC loaded with a specific peptide antigen; the second one is provided by the interaction of the T-cell co-stimulatory receptor CD28 with its ligands, B7-1 (CD80) and (CD86) B7-2, expressed on the surface of APC. In resting naïve T cells, CTLA-4, which is a CD28 homolog with much higher affinity for B7, localizes primarily in the intracellular compartment [178, 179]. Stimulatory signals resulting from both TCR/MHC and CD28/B7 binding induce exocytosis of CTLA-4 from CTLA-4-containing vesicles, and its translocation to the T-cell surface. Here, CTLA-4 outcompetes CD28 in its binding to B7-1 and B7-2. Maximal CTLA-4 expression on the T-cell surface is reached within 2–3 days from the stimulatory signal, allowing the immune system to be operative in the first phase and to be blocked when the threat is likely defeated [1, 178–180]. In fact, CTLA4/B7 transmits an inhibitory signal to T cells that downregulates IL-2 production, and reduces the proliferation rate and survival of activated T cells [179–181]. The relative amount of the CD28/B7 complex versus the CTLA-4/B7 one determines whether a T cell will undergo activation or anergy (i.e., T-cell non-responsiveness in response to an antigen). Blockade of CTLA-4 in T-lymphocytes hampers the inhibitory signal that derives from its binding to B7 expressed on APC membranes [179–181]. Therefore, anti-CTLA-4 antibodies favour the reactivation of T lymphocytes during the phase of antigen presentation by APCs, stimulating a more robust antitumour response [1, 2, 178].

Whilst CTLA-4 is operative during the priming phase of T-cell activation, the programmed death (PD-1) pathway (a member of the CD28 family of receptors) acts during the effector phase, dampening cell activation predominantly within peripheral tissues [47, 182]. The expression of PD-1 is transcriptionally induced

upon T-cell activation, whereas it is absent on naïve T cells. PD-1 regulates T-cell activation through the binding to its ligands, PD-L1 and PD-L2. Even though CTLA-4 and PD-1 seem to cover similar inhibitory effects on T-cell activity (e.g., inhibition of T-cell proliferation and survival, reduction in IL-2 and IFN- γ release), these pathways differ in their timing and tissue localization [47, 182]. Remarkably, PD-1 expression (together with other ICK) is a hallmark of “exhausted” T cells (see throughout the text), a condition characterized by T-cell dysfunction occurring during chronic infections and cancer, which results in a suboptimal control of these threats [183–185]. PD-L1 and PD-L2 are widely expressed in healthy cells (PD-L1 is primarily expressed by leukocytes, hematopoietic cells, and non-lymphoid tissues, whereas PD-L2 is detected in DCs and monocytes) and, especially PD-L1, in a wide variety of tumour cells. After PD-L1 or PD-L2 engagement, PD-1 primarily transmits a negative co-stimulatory signal in order to limit T-cell activation through the recruitment of the tyrosine phosphatase SHP2, which attenuates TCR signalling [183]. Remarkably, SHP2 leads to preferential de-phosphorylation of CD28 rather than TCR. This phenomenon, along with the CTLA-4 inhibition of CD28 signalling discussed above, might represent a functional convergence point between CTLA-4 and PD-1 along the CD28 pathway [182]. Blockade of PD-1 signalling prevents PD-1-mediated attenuation of TCR signalling and allows the reactivation of exhausted CD8⁺ effectors. Therefore, despite continued PD-L1 ligand expression within TME, exhausted T cells are able to mount an effective immune response towards tumour cells [182–185]. Since CTLA-4 inhibition hampers the induction phase of antitumour T-cell immunity in lymphoid tissues, whereas PD-1 inhibition primarily affects the effector phase within the TME, the

dual blockade of both ICKs results in additive/synergistic effects [1, 2, 186].

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conceptualization: G.R.T., D.S., and G.G.; writing of original draft preparation: G.R.T., D.S., G.G.; critical review of the manuscript: P.M.L.; review and editing, G.G. and S.M.

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