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Review

Negative immune checkpoints on T lymphocytes and their relevance to cancer immunotherapy☆

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ABSTRACT

The term 'inhibitory checkpoint' refers to the broad spectrum of co-receptors expressed by T cells that negatively regulate T cell activation thus playing a crucial role in maintaining peripheral self-tolerance. Co-inhibitory receptor ligands are highly expressed by a variety of malignancies allowing evasion of anti-tumour immunity. Recent studies demonstrate that manipulation of these co-inhibitory pathways can remove the immunological brakes that impede endogenous immune responses against tumours. Antibodies that block the interactions between co-inhibitory receptors and their ligands have delivered very promising clinical responses, as has been shown by recent successful trials targeting the CTLA-4 and PD-1 pathways. In this review, we discuss the mechanisms of action and expression pattern of co-inhibitory receptors on different T cells subsets, emphasising differences between $CD4^+$ and $CD8^+$ T cells. We also summarise recent clinical findings utilising immune checkpoint blockade.

1. Introduction

T cell-mediated immunity is tightly regulated by a series of complex mechanisms that range from the elimination of self-reactive T cell clones in the thymus to the fine-tuning of T cell activation by co-receptors. Co-stimulatory and co-inhibitory receptors demonstrate great diversity in structure, function and expression pattern, and their functions are largely context dependent. They regulate a broad spectrum of T cells activity from proliferation and motility to the expression of cytokines and cytotoxic molecules, such as granzyme B and perforin. Co-inhibitory receptors play a crucial role in curtailing T-cell activation and defects in their function lead to aberrant immune responses such as autoimmunity. The same regulatory mechanisms limiting T cell activation following chronic exposure to antigen or preventing autoimmune responses may, however, diminish adequate immune responses against cancer. Supported by pre-clinical and

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clinical evidence, novel immunotherapeutic strategies have focused on monoclonal antibodies designed to block the interaction between co-inhibitory receptors expressed on T cells and their respective ligands, with the aim of increasing the anti-tumour immune response. Optimisation of these therapies has evolved thanks to the understanding of the function of the various checkpoints, their expression landscape and their effect on both the effector and regulatory T cell (Treg) subpopulations prevalent in many malignancies.

In this review, we specifically focus on the role of negative immune checkpoints in T cell responses, the adaptive immune resistance and the complex interplay between the receptors and their multiple ligands in the tumour microenvironment. We highlight the differences in co-inhibitory receptor expression in different subsets of $CD4^+$ and $CD8^+$ T cells and summarise both early clinical experience with the use of monoclonal antibodies and the outcomes of late-stage clinical trials with anti-CTLA-4 and anti-PD-1 antibodies.

2. Inhibitory receptors: mechanism of action and targeted therapies

2.1. 1 CTLA-4

2.1.1. *CTLA-4 structure, endo- and exocytosis, mechanism of action*

CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, CD152) is a CD28 homologue and genes for these molecules are localised in the same chromosome regions in human and mouse. The overall homology between the human and murine CTLA-4 proteins is 76%, whereas cytoplasmic domains show complete identity. CTLA-4 is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily (IgSF) and exhibits a dimer interface similar to that present in the constant (c-type) family members (Dariavach et al., 1988; Ostrov et al., 2000). The full length CTLA-4 consists of 4 exons, in humans in addition transcripts skipping exon 2–3 can be detected and in mouse transcripts lacking exon 2 encoding ligand-binding domain were described (Ling et al., 1999).

CTLA-4 is expressed mainly by T cells. Some studies report its expression on a variety of immune cells including B cells, fibroblasts and embryonic cells, but the role of CTLA-4 remains unknown for these non-T cell subsets (Quandt et al., 2007). Whereas both human and murine natural regulatory T cells (nTreg) cells constitutively express high levels of CTLA-4 on their surface, naive T cells upregulate CTLA-4 only after activation, reaching the maximum level 2–3 days post *in vitro* activation with anti-CD3 antibodies (Walunas et al., 1994). In non-activated T cells, expression of CTLA-4 is practically undetectable (Perkins et al., 1996). Upon activation *in vitro*, both $CD4^+$ and $CD8^+$ T cells express CTLA-4, although $CD4^+$ T cells are reported to express more CTLA-4 than $CD8^+$ T cells on both the mRNA and protein level (Table 1). Unfortunately it is not clear if Foxp3-positive cells were excluded from these analyses (Chan et al., 2014). Although CTLA-4 upregulation upon TCR engagement is well described, the transcription factors driving its expression are not completely elucidated. Indeed, only NFATc1 (nuclear factor for activated T cells) has been reported to bind CTLA-4 promoter in human primary T lymphocytes (Gibson et al., 2007; Chan et al., 2014). The mechanisms regulating surface expression level of CTLA-4 are better understood: exocytosis and endocytosis. CTLA-4 is mainly located in intracellular vesicles and interacts with the

Table 1 – Simplified expression of inhibitory molecules on T cells.

			CTLA-4	PD-1	TIGIT	VISTA	LAG-3	TIM1	TIM3	CEACAM1	LAIR-1	HVEM	BTLA	CD160	CD200	CD200R	A2ar
CD8	human	naïve	none	none	ND	low	low	none	none	ND	high	high	high	medium	medium	ND	none
CD4 eff	human	naïve	none	none	none	low	low	low	none	ND	high	high	high	low	high	ND	none
Treg	human	naïve	high	none	low	low	medium	ND	none	ND	high	ND	ND	ND	high	ND	none
CD8	murine	naïve	none	none	low	medium	low	none	none	ND	medium	high	low	low	ND	ND	none
CD4 eff	murine	naïve	none	none	low	medium	low	low	none	ND	medium	high	low	low	ND	ND	none
Treg	murine	naïve	high	none	ND	medium	medium	ND	none	ND	medium	high	low	ND	ND	ND	none
CD8	human	resting	ND	low	low	ND	ND	none	none	none	ND	ND	ND	ND	medium	high	ND
CD4 eff	human	resting	ND	low	low	ND	ND	none	none	low	ND	ND	ND	ND	medium	high	ND
Treg	human	resting	ND	low	medium	ND	ND	none	none	low	ND	ND	ND	ND	medium	high	ND
CD8	murine	resting	ND	low	low	medium	ND	none	none	low	ND	ND	low	ND	ND	low	ND
CD4 eff	murine	resting	ND	low	low	medium	ND	none	none	low	ND	ND	low	ND	ND	medium	ND
Treg	murine	resting	ND	low	medium	ND	ND	none	none	low	ND	ND	low	ND	ND	medium	ND
CD8	human	activated	medium	medium	medium	medium	medium	none	medium	ND	medium	med/high	medium	high	high	high	low
CD4 eff	human	activated	medium	medium	medium	medium	medium	medium	low	ND	medium	med/high	high	low	high	high	medium
Treg	human	activated	high	medium	high	medium	high	ND	high	ND	medium	ND	ND	ND	high	high	ND
CD8	murine	activated	medium	high	ND	medium	medium	none	high	medium	high	medium	high	high	ND	ND	low
CD4 eff	murine	activated	medium	high	ND	medium	medium	medium	medium	medium	high	medium	high	low	ND	ND	medium
Treg	murine	activated	high	high	ND	medium	high	ND	high	medium	high	high	medium	ND	ND	ND	ND
CD8	human	exhausted	medium	high	ND	medium	ND	none	high	ND	ND	ND	ND	high	ND	ND	ND
CD4 eff	human	exhausted	medium	high	ND	medium	ND	high	high	ND	ND	ND	ND	ND	ND	ND	ND
Treg	human	exhausted	high	high	ND	medium	ND	ND	high	ND	ND	ND	ND	ND	ND	ND	ND
CD8	murine	exhausted	medium	high	high	medium	high	none	high	medium	ND	ND	ND	high	ND	ND	ND
CD4 eff	murine	exhausted	medium	high	high	medium	medium	high	high	medium	ND	ND	ND	ND	ND	ND	ND
Treg	murine	exhausted	high	high	ND	medium	ND	ND	high	ND	ND	ND	ND	ND	ND	ND	ND
CD8	human	memory	ND	high	ND	low	ND	ND	high	ND	medium	ND	ND	high	ND	ND	ND
CD4 eff	human	memory	ND	high	high	low	ND	ND	high	ND	medium	ND	ND	ND	ND	ND	ND
Treg	human	memory	ND	ND	high	low	ND	ND	ND	ND	medium	ND	ND	ND	ND	ND	ND
CD8	murine	memory	ND	high	ND	medium	medium	ND	high	ND	high	ND	ND	high	ND	ND	ND
CD4 eff	murine	memory	ND	high	ND	medium	medium	ND	high	ND	high	ND	ND	ND	ND	ND	ND
Treg	murine	memory	ND	ND	ND	medium	ND	ND	ND	ND	high	ND	ND	ND	ND	ND	ND

μ2 subunit of the clathrin adaptor protein complex AP2 (Follows et al., 2001). Redistribution of CTLA-4 to the plasma membrane is dependent on the GTPase ADP ribosylation factor-1 and on phospholipase D activity as inhibition of these pathways prevents its membrane expression (Mead et al., 2005). Endocytosis (internalisation) of CTLA-4 utilises a clathrin- and dynamin-sensitive pathway and it is not impaired during T cell activation. CTLA-4 is degraded in lysosomes (Mead et al., 2005).

Being a CD28 homologue, CTLA-4 also binds to the same ligands as this co-stimulatory receptor. However, CTLA-4 interacts with CD80 and CD86 with 10 times higher affinity than CD28. Furthermore, CD28 recruitment to the immunological synapse can be disrupted by CTLA-4, which forms extended high affinity lattices of alternating CTLA-4 and CD80 homodimers (Greene et al., 1996). Thus, CTLA-4 competes with CD28 ligands and thereby prevents CD28-mediated T cell activation. Both ligands are expressed by antigen presenting cells (APCs). CD86 is constitutively expressed and upregulated after activation while CD80 is expressed only after activation (Hathcock et al., 1994). Mice that are deficient for either CD80 or CD86 show that these molecules have partially overlapping function. So far, there are no reports suggesting any unique biochemical or molecular differences in interactions of CTLA-4 with either of the ligands. Despite extensive studies, little is known about the intracellular signalling pathways initiated upon CTLA-4 engagement by its ligands. In a sharp contrast to CD28 or ICOS, engagement of CTLA-4 *in vitro* leads to only relatively small changes in the transcriptional profile (Wakamatsu et al., 2013). In agreement with these results, the Allison team showed that only 9 genes besides CTLA-4 itself were differentially expressed between CTLA-4 sufficient and deficient T cells upon *in vivo* antigenic stimulation (Corse and Allison, 2012). These data suggest that there is no obvious inhibitory signalling pathway initiated by the engagement of CTLA-4 (further reading: (Walker and Sansom, 2015)). A recent report focusing on Treg cells showed that the CTLA-4 cytoplasmic tail interacts with the protein kinase C-η (PKC-η) in this T cells subset and that PKC-η-deficient Treg cells were impaired in contact-dependent suppressive activity, which was associated with a grossly defective activation of the transcription factors NFAT and NF-κB in these cells. In addition, this study demonstrated that CTLA-4/PKC association mediates recruitment of focal adhesion disassembly complex (GIT2-aPIX-PAK) and hence plays a role in T cell motility (Kong et al., 2014; Walker and Sansom, 2015).

In 2011, Qureshi et al. characterised the cell-extrinsic function of CTLA-4. They showed that CTLA-4 captures CD80 (B7-1) and CD86 (B7-2) from neighbouring cells by a unidirectional process called trans-endocytosis. With a mutant lacking the conserved C-terminus domain of CTLA-4, they defined the interaction involved in this process. The acquisition of CD80 and CD86 by CTLA-4 was enhanced upon TCR stimulation. Interestingly, *in vivo* data showed that both $Foxp3^+$ and $Foxp3^-$ are capable of trans-endocytosis (Qureshi et al., 2011).

2.1.2. *CTLA-4, tumour immunity: pre-clinical data*

Numerous studies with different disease models show that CTLA-4 is a crucial molecule for T cell homoeostasis and function, but is also vital for maintaining peripheral tolerance. CTLA-4-deficient mice suffer from early onset aggressive autoimmune diseases with multi-organ lymphocytic infiltration and organ destruction and in consequence premature death by 3–4 weeks of age (Tivol et al., 1995). Further studies with CTLA-4 KO mice show that CTLA-4 may have different impact on $CD4^+$ vs. $CD8^+$ T cells homoeostasis and function. In this model CTLA4-deficient $CD8^+$ T cells do not get activated and expand when CTLA-4 KO $CD4^+$ T cells are depleted but CTLA-4 KO $CD4^+$ T cells do in the absence of CTLA-4 KO $CD8^+$ (Chambers et al., 1997). Similar results are found in human T cells: blocking of CTLA-4 *in vitro* on T cells results in a significant increase in proliferation of $CD4^+$ but not $CD8^+$ T cells (Chan et al., 2014). Nonetheless, despite the lack of evidence supporting a relevant role for CTLA-4 on primary CD8 responses, CTLA-4 has been demonstrated to modulate secondary responses in $CD8^+$ T cells (Chambers et al., 1998).

High levels of CTLA-4 expression on regulatory T cells suggested that CTLA-4 may play a crucial role in Treg-mediated suppression. One of the major functions of Treg cells is the inhibition of priming and differentiation of effector T cells (Josefowicz et al., 2012). Among many mechanisms employed by Treg cells, CTLA-4-mediated suppression is considered to be the most crucial one *in vivo*. The absence of CTLA-4 specifically on Treg cells (Foxp3-Cre × $CTLA\text{-}4^{fl/fl}$ model) is sufficient for the development of systemic autoimmune disease, which confirms the importance of CTLA-4 inhibiting overt immune response (Kajsa, 2008). These genetically modified regulatory T cells were not able to suppress *in vivo* tumour rejection, leading to enhanced tumour immunity (Kajsa, 2008).

Data from numerous *in vitro* and *in vivo* experiments demonstrate that CTLA-4 is a negative regulator of T-cell mediated responses in tumours. The first successful attempt at blocking the CTLA-4 pathway to increase anti-tumour immunity was reported by J. Allison's group in 1996 where *in vivo* administration of anti-CTLA-4 antibody induced the rejection of established murine colon carcinoma (Leach et al., 1996). Subsequently, anti-CTLA-4 treatment was tested in several highly immunogenic murine tumour models including prostatic carcinoma, lymphoma, and renal carcinoma (Kwon et al., 1997b). Anti-CTLA-4 treatment was shown to enhance anti-tumour responses by $CD8^+$ OT-I cells against EG.7 Ova-expressing tumours. The effect appeared to be dependent on $CD4^+$ T cells (Shrikant et al., 1999). Studies with gp100-specific TCR transgenic mice (Pmel) crossed to the CTLA-4 KO strain also confirmed that autoimmunity and tumour immunity mediated by these $CD8^+$ T cells required CTLA-4 –deficient $CD4^+$ T cells (Gattinoni et al., 2006).

As a monotherapy, anti-CTLA-4 mAbs failed to promote rejection of established poorly immunogenic tumours leading to a number of studies assessing potential additive or synergistic activity in combination with other approaches. Both conventional approaches such as radio and chemotherapy as well as immune modulatory interventions targeting innate and adoptive immunity were proposed and evaluated (Peggs et al., 2008). Successful outcome brought studies that combined anti-CTLA-4 treatment with administration of cytokines that were reported to enhance T cell priming, infiltration of innate cells to tumour or INF-γ expression. CTLA-4-blockade in combination with granulocyte-macrophage colony stimulating factor (GM-CSF) – expressing tumour cell-based vaccine

(GVAX) proved to be very efficient in treatment of established tumour e.g. B16 melanoma (Quezada et al., 2006; van Elsas et al., 1999). The GVAX/anti-CTLA-4 combined treatment was also shown to elicit potent immune responses that decreased the incidence of prostate cancer in TRAMP mice (Hurwitz et al., 2000). Increased T cell-mediated immunity was also described when intratumoural IL-12 application was combined with systemic blockade of CTLA-4 to cure established Gl261 glioblastoma (Vom Berg et al., 2013).

Despite much investigation, the precise mechanisms underpinning the *in vivo* activity of anti-CTLA-4 mAbs have remained elusive. Whilst the prevailing hypothesis had been that CTLA-4-blockade "released the breaks" on effector T cells, the demonstration that the antibody needed to target both Teff (effector T cells) and Treg for maximal anti-tumour activity supported the potential existence of an additional mechanism of action (Peggs et al., 2009). Most recently, it was demonstrated that in addition to its immune-modulatory activity on effector T cells, anti-CTLA-4 also promoted site-dependent Treg depletion *in vivo*. Depletion was restricted to the tumour and mediated by macrophages in the tumour expressing high levels of FcγRIV (Simpson et al., 2013). Interestingly, depletion was not restricted to Tregs but to cells expressing high levels of CTLA-4 on their surface. Both $CD8^+$ and $CD4^+Foxp3^-$ cells expressed lower levels of surface CTLA-4, hence favouring depletion of tumour infiltrating Tregs, which express high levels of surface CTLA-4 (Simpson et al., 2013). Similar observations for other tumour models were published by Selby et al.; the authors showed that only a mIgG2a anti-CTLA-4 mAb with capacity for ADCC (antibody-dependent cell-mediated cytotoxicity) was able to deplete Tregs-infiltrating CT26 and M38 tumours but that efficacy was lost once the Fc portion of the mAb was engineered to a mIgG1 isotype with reduced ADCC capacity ((Selby et al., 2013); further reading: (Furness et al., 2014)). Thus, it has been become clear that even if the target of immunotherapy is solely expressed by T cells, the role of tumour microenvironment cannot be underestimated. Characterisation of innate cells infiltrating tumour, especially effector macrophages in terms of expression of Fc receptors will likely be necessary to better understand and predict the outcome of treatment with antibody of a certain isotype.

2.1.3. *Anti-CTLA4 antibody based therapies. Clinical trials with ipilimumab and tremelimumab*

Two fully human monoclonal anti-CTLA-4 antibodies have entered clinical trials: ipilimumab (MDX-010, Medarex) and tremelimumab (Pfizer). Up to 42 studies for clinical trials phase I/II and 3 phase III clinical trials with ipilimumab as a single agent or with combined therapies have been completed (http://clinicltrials.gov) (Table 2).

Early phase studies with ipilimumab in previously vaccinated metastatic melanoma and ovarian carcinoma patients (Hodi et al., 2003) demonstrated tolerability and suggested that ipilimumab could amplify a long-lived memory response in patients, thus justifying further evaluation. Another pilot study using ipilimumab as a monotherapy in patients with metastatic hormone-refractory prostate cancer showed decrease in prostate-specific-antigen (PSA) level $\geq$ 50% in two out of fourteen patients (Small et al., 2007); for details about phase I and II clinical trials please see review: (Peggs et al., 2008).

In the first randomised phase III controlled trial of ipilimumab 676 patients with unresectable stage III or IV melanoma were recruited to test its activity as a monotherapy or in combination with a peptide vaccine targeting the melanoma associated antigen gp100 (glycoprotein 100). Patients were randomly assigned in a 3:1:1 ratio to receive ipilimumab (3 mg/kg) plus gp100, or single agent ipilimumab or gp100. The median overall survival was 10.1 months among patients receiving ipilimumab compared to 6.4 months in those receiving gp100 alone (HR 0.66; $p = 0.003$). The response rate was significantly higher in the ipilimumab monotherapy group compared to the gp100 group (10.9% versus 1.5%; $p = 0.001$). The responses to ipilimumab were durable with the 1-year and 2-year survival rates being 46% and 24%, respectively, in the ipilimumab monotherapy group compared to 25% and 14%, respectively, in the gp100 group (Hodi et al., 2010) (Table 2). In keeping with murine studies in CTLA-4 deficient mice and phase I/II clinical trials, immune-related adverse effects were observed in up to 60% of the patients, most of them being reversible when appropriate treatment with steroids or other immunosuppressive drugs applied. The outcome of that trial was the basis for the approval of ipilimumab for the treatment of advanced melanoma by the FDA in 2011 and in 2013 by EMA.

Another phase III clinical trial in metastatic melanoma evaluated ipilimumab monotherapy against ipilimumab with dacarbazine treatment (chemotherapy) in previously untreated patients. The combination therapy resulted in higher ORR (>15%) than the dacarbazine treatment only (Robert et al., 2011). Ipilimumab was also tested in melanoma patients with brain metastasis in a phase II clinical trial. Ipilimumab showed activity in some patients particularly when metastases were small and asymptomatic. No unexpected toxic effects in this population were observed (Margolin et al., 2012) (Table 2).

Despite promising results from phase I/II clinical trials with tremelimumab (monotherapy) the phase III randomised trial with advanced melanoma patients failed to demonstrate a statistically significant survival advantage of treatment with tremelimumab over standard-of-care chemotherapy in first-line treatment of patients with metastatic melanoma (Ribas et al., 2013). On the basis of pre-clinical studies one may hypothesise that the lack of ADCC capacity shown by tremelimumab (IgG2 isotype) could be one of the reasons of its inferior activity to ipilimumab. However, several other points need to be considered: the unexpectedly good outcome in the control arm, possibly due to the enrolment of patients with a more favourable prognosis and use of another CTLA4-blocking agent (ipilimumab) as salvage therapy for patients in the comparator arm (Ribas et al., 2013).

Clinical trials with CTLA-4 blockade suggested that conventional Response Evaluation Criteria in Solid Tumours (RECIST) might be not effective as a method for evaluating patients undergoing immune-modulatory treatment. In initial phases of treatment some patients who subsequently achieved disease response presented with increased tumour growth due to the ongoing inflammatory reaction. Therefore additional criteria have been proposed (immune-related

Table 2 – Clinical trials targeting inhibitory checkpoints pathways.

Target	Antibody/Isotype/ Activity	Disease	Therapy (Mono or combained)	Phase/Owner/ Sponsor	No. patients	Results	Adverse events and immune-related adverse events (irAEs)	Reference
CTLA-4	Ipilimumab (IgG1κ, human), Antagonist	Stage IV melanoma	Monotherapy	III (NCT00094653)/ Bristol-Myers Squibb	676	ORR: 10,9%; OS: ipilimumab + gp100: 10.0 months, gp100: 6.4 months	60% (ipilimumab) 32% (gp100) Grade 3–4: 10–15% (ipilimumab) 3% (gp100) Dermatological,gastrointestinal, endocrine and hepatic side effects	Hodi et al., 2010
		Melanoma with brain metastasis	Monotherapy	II (NCT00623766)/ Bristol-Myers Squibb/Medarex	72	Disease control within the brain: 24% of patients in Cohort A (neurologically asymptomatic patients) and 10% of patients B (symptomatic patients receiving systemic corticosteroids); OS at 1 year (A) 31%, (B) 19%, at 2 years (A) 26% (B) 10%	Grade 3–4 similar in both cohorts: fatigue, diarrhoea, nausea, headache, rash and pruritus	Margolin et al., 2012
		Stage IV melanoma	Combined (dacarbazine)	III (NCT00324155)/ Bristol-Myers Squibb/Medarex	502	ORR: 15.2%; OS: ipilimumam + dacarbazine: 11.2 months vs. dacarbazine 9.1 months, OS at 1 year, respectively (47.3% vs. 36.3%), 2 years (28.5% vs. 17.9%), and 3 years (20.8% vs. 12.2%)	Similar to prior studies with ipilimumab, except higher rate for liver-function and lower rats for gastrointestinal	Robert et al., 2011
		Metastatic castration-resistant prostate cancer	Monotherapy	III (NCT00861614)/ Bristol-Myers Squibb	799	No significant effect in terms of overall survival	Grade 3–4: diarrhoea (16% of patients in the ipilimumab group vs 2% in the placebo group), fatigue (11% vs 9%), anaemia (10% vs 11%), and colitis (5%vs 0). 4 (1%) deaths from toxic effects of the ipilimumab	Kwon et al., 2014
	Tremelimumab (IgG2, human), Antagonist	Stage IV melanoma	Monotherapy	III (NCT00257205)/ Pfizer	655	ORR: 10.7%; OS: tremelimumab: 12.6 months, chemotherapy: 10.7 months	Diarrhoea, pruritus and rash in the tremelimumab group, endocrine toxicities: 7.4%, 7 deaths in the tremelimumab group	Ribas et al., 2013

Anti-PD-1	Nivolumab (IgG4 fully human), Antagonist	Advanced renal carcinoma	VEGFR TKIs (98%) mTOR inhibitors (38%) and immunotherapy (24%)	II (NCT01354431)/ Bristol-Myers Squibb	168	19/35 responders (54%) OR lasting >12–20 + months/ Median OS 18.2 months/4.2 months (10 mg kg−1)	Grade 3–4: 17%	Motzer RJ, J Clin Oncol (Meeting Abstracts) 32:5s.
		Metastatic melanoma (MM)	Monotherapy	III (NCT01721772)/ Bristol-Myers Squibb	418	ORR nivolumab group: 40.0%, dacarbazine group: 13.9% At 1 year, OS nivolumab group: 72.9%, dacarbazine group: 42.1% in the/PFS nivolumab group: 5.1 months, dacarbazine group: 2.2 months	Grade 3–4: nivolumab group 11.7%, dacarbazine group 17.6%	Robert et al., 2015
		Metastatic melanoma	Nivolumab (n = 268) or ICC = dacarbazine or carboplatin + paclitaxel (n = 102)	III (NCT01721746) open-label trial/ Bristol-Myers Squibb	268	ORR 32%/median time to response of 2.1Reduction of ≥50% in target lesion burden occurred in 82% (31/38) of nivolumab responders and 60% (3/5) of responders	Grade 3–4: 9%	Weber JS et al. Eur Soc Med Oncol Congr (Meeting Abstracts) 728.
	Pembrolizumab (Humanised IgG4), Antagonist	MM ipilimumab refractory	Monotherapy	II (NCT01295827)/ Merck Sharp & Dohme Corp.	411	ORR: 34%, PFS: 5.5 months, OS: 69% at one year and 62% at 18 months		Ribas A, Wolchok JD et al. Soc Melanoma Res (Meeting Abstracts).
	Pidilizumab (CT-011, Humanised IgG1k), Antagonist	Metastatic melanoma	Monotherapy	II (NCT01435369)/ CureTech Ltd	103	ORR prior Ipi: 10.0% vs 5.9%, PFS prior Ipi: 2.8 vs 1.9 months, OS at 1 year 64.5%, without Ipi 55.7% B-RAF V600 WT tumours 69.3%	Fatigue (43%), diarrhoea (22.5%), arthralgia (21%) pneumonia (5%) and dyspnoea (3%)	Atkins et al. J Clin Oncol 32:5s, 2014 (suppl; abstr 9001)
		Follicular lymphoma	Rituximab	II (NCT00904722)/ M.D. Anderson Cancer Center	29	ORR 66%, PFS 21.1%; complete responses in 15 (52%) patients and partial responses in 4 (14%)	Grade 3–4: 0%	Westin et al., 2014
CD200	Samalizumab (ALXN6000, Humanised IgG2/G4 κ), Antagonist	Advanced CLL or Multiple Myeloma	Monotherapy	I/II (NCT00648739)/ Alexion pharmaceutics	26	19/20 with 81%–98% reduction in peripheral CD200+ CD4+ T cells, (14/21) with 64%–75% CD200 loss on B-CLL cells. Overall, 36% (8/22) of evaluable patients experienced at least a 10% reduction in bulky disease	Grade 3–5: anaemia and neutropenia (8%), thrombocytopaenia, reduced visual acuity, respiratory syncytial virus infection and rash (4%)	Alexander, 2011 P T. 2011 Feb; 36(2): 100–103 (Meeting abstract)

response criteria (irRC)) that consider total tumour burden regardless of the growth of new disease, with higher maximum tumour growth allowed within the bounds of stable disease (Weber et al., 2012).

Although clinical trials with ipilimumab show promising results the exact mechanism of its action is still not completely resolved. Analysis of PBMC from four NY-ESO-1 seropositive melanoma patients treated with ipilimumab demonstrated an expansion of NY-ESO-1 antigen-specific cytotoxic $CD4^+$ T cells that show increased granzyme B and perforin production (Kitano et al., 2013). Recently the analysis of innate cells isolated from peripheral blood from the patient with advanced melanoma treated with ipilimumab revealed an increase of nonclassical $CD14^+CD16^{++}$ monocytes in responding patients. These cells expressed high level of FcγRIIIa and were able to lyse Treg cells *in vitro* in the presence of ipilimumab (Romano et al., 2015). Earlier reports showed that patients treated with anti-CTLA-4 do not have decreased numbers of regulatory T cells when changes in peripheral blood were studied (Maker et al., 2005). Clearly further investigations are required to explain the mechanistic basis of anti-CTLA-4 therapies in human malignancies. Since most of the work done so far has focused on peripheral blood, a thorough analysis of tumour-infiltrating immune cells will be crucial for better understanding of the mechanisms of anti-CTLA-4-mediated therapy. To be able to better predict the *in vivo* activity of immune-modulatory antibodies in cancer patients, analysis of target molecule density on tumour-infiltrating T effector and Treg cells rather than cells present in peripheral blood will likely be vital. Moreover, the different outcomes of clinical trials with anti-CTLA-4 antibodies of different isotypes highlight the importance of FcγR-expressing innate cells in antibody-mediated immunotherapies.

2.2. *The PD-1/PD-L1 axis*

2.2.1. *PD-1 expression*

First identified as a protein upregulated in a murine T-cell hybridoma undergoing programmed cell death (Ishida et al., 1992), PD-1 is an IgSF member. PD-1 possesses an N-terminal IgV-like domain, a transmembrane domain and a cytoplasmic tail containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 is a monomeric molecule on the cell surface lacking the extracellular cysteine found in CD28, CTLA-4, and ICOS, which allows these proteins to homodimerise (Zhang et al., 2004). PD-1 is absent on naïve cells but is highly expressed on activated T cells, B cells (Good-Jacobson et al., 2010), NK cells (Terme et al., 2011) and myeloid-derived cells (Keir et al., 2008). Its expression can be induced by TCR signalling (Agata et al., 1996) and by the common γ chain cytokines (interleukin-2 (IL-2), IL-7, IL-15, and IL-21) (Kinter et al., 2008). Recently, NFATc1 binding site was identified in the PD-1 promoter region making NFATc1 a critical factor for PD-1 expression on T cells (Oestreich et al., 2008).

2.2.2. *PD-L1/PD-L2 expression*

Like PD-1, its two known ligands PD-L1 (programmed cell death protein 1 ligand 1; B7H1 and CD274) (Dong et al., 1999) and PD-L2 (B7DC and CD273) (Latchman et al., 2001; Tseng et al., 2001) display a complex expression pattern. PD-L1 is constitutively expressed at low levels and further upregulated upon cell activation on both haematopoietic cells, including T, B, myeloid, and dendritic cells, and non-hematopoietic cells as in the lung, heart, and especially on a wide range of cancer cells (Chen et al., 2012a; Liang et al., 2003; Rodig et al., 2003; Sznol and Chen, 2013). PD-L1 can be upregulated on tumours by activation of key oncogenic pathways [phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK)] or by IFN-γ production in the tumour microenvironment by T-cell responses (Pardoll, 2012; Parsa et al., 2007; Taube et al., 2012). PD-L1 hampers anti-tumour immunity by tolerising tumour-reactive T cells through PD1 interaction on activated T cells (Dong et al., 2002; Latchman et al., 2004), by rendering tumour cells resistant to $CD8^+$ T cell-mediated destruction and refractory to apoptosis induced by Fas ligation (Azuma et al., 2008), by promoting the development and maintenance of induced T regulatory cells (Francisco et al., 2009), and by tolerising T cells by reverse signalling through T cell–expressed CD80 (Butte et al., 2007; Park et al., 2010). Interestingly, the bidirectional inhibitory interaction reported between PD-L1 and CD80 demonstrate that CD80 may serve as a biomarker and functional checkpoint for T cell anergy. Whether this interaction is involved in the immune evasion by tumour cells needs to be further investigated in human cells. Nevertheless, a role of CD80 on T cells as an inhibitory receptor to deliver outside-in signal is concordant with previous findings in mice, including: increased cytokine productions in CD80-KO T cells (Schweitzer and Sharpe, 1999), an enhanced severity of graft-versus-host disease by CD80/CD86-KO donor T cells (Taylor et al., 2004), as well as resistance of CD80-KO T cells to inhibitory effects of Tregs (Paust et al., 2004).

PD-L2 has a distinct expression pattern and was initially thought to be restricted to macrophages and dendritic cells (DCs) (Latchman et al., 2001). Recently, however, PD-L2 expression has been extended to some solid tumours, such as ovarian carcinoma (Hamanishi et al., 2007), small cell lung cancer (Konishi et al., 2004), Oesophageal cancer (Ohigashi et al., 2005) and tumour-associated-fibroblast, enhanced by treatment with IFN-γ (Nazareth et al., 2007). Furthermore, PD-L2 has also been found on activated $CD4^+$ and $CD8^+$ T cell subsets that bring an additional level of complexity. Engagement of PD-L2, predominantly expressed on T helper type 2 cells (Th2), was able to down-modulate cytokine production and cell proliferation (Messal et al., 2011; Lesterhuis et al., 2011). It has been demonstrated that PD-L1 and PD-L2 cross-compete to bind to PD-1 (Ghiotto et al., 2010) although the relative affinity of PD-L2 to PD-1 is 2–6-fold higher than that of PD-L1 (Youngnak et al., 2003). However, PD-L2 is generally expressed at a lower level, which favours PD-L1 as the primary binding ligand of PD-1, except for Th2 responses.

2.2.3. *PD-1 signalling pathway-molecular mechanisms*

It was demonstrated that the signalling pathway initiated by interactions of PD-1 with its ligands (PD-L1, PD-L2) depends on the phosphorylation of two tyrosines at the intracellular tail of PD-1. The recruitment of SH2-domain containing

protein tyrosine phosphatases (SHP-1 and/or SHP-2) to the ITSM cytoplasmic region of PD-1 then inhibits downstream signals of the T-cell receptor, particularly PI3K/AKT activation (Riley, 2009; Okazaki et al., 2001; Plas et al., 1996). Genetic ablation of PD-1 in mice has highlighted its crucial function in maintaining peripheral tolerance and T cell-exhaustion. PD-1-deficient mice present various autoimmune conditions, including autoantibody-induced cardiomyopathy (Nishimura et al., 2001; Okazaki et al., 2003), arthritis and lupus-like disease (Nishimura et al., 1999) and type 1 diabetes (Wang et al., 2005; Yao et al., 2013). In the periphery, these suppressive functions of PD-1 seem to be mediated by PD-L1 (Ansari et al., 2003; Tsushima et al., 2007). This crucial function of the PDL-1/PD-1 axis in the control of human T cell activation can be exploited by tumour cells (Zou and Chen, 2008). Indeed, tumour-induced PD-Ls utilise several mechanisms to suppress and facilitate the evasion of host immune surveillance, including the promotion of T cell anergy and exhaustion (Crespo et al., 2013), unresponsiveness and apoptosis (Dong et al., 2002) and inducing the expansion of Treg cells (Amarnath et al., 2011; Wang et al., 2008). Moreover, upon PD-1 ligation, PD-L1 expressing tumours can receive an anti-apoptotic signal, which renders them resistant to lysis by cytotoxic T lymphocytes (CTLs) and FAS-induced apoptosis (Azuma et al., 2008; Dong et al., 2002; Hirano et al., 2005; Yao and Chen, 2006).

2.2.4. *PD-1 pathway blockade and clinical relevance*

PD-1 blockade *in vivo* by specific mAbs promotes CTLs expansion (Strome et al., 2003) and can reduce tumour growth or induce spontaneous tumour rejection (Blank et al., 2004; Crespo et al., 2013; Kryczek et al., 2006). PD-1 blockade is clinically relevant since exhausted PD-1^{+}CD8^{+} T cells have been found in patients with melanoma (Baitsch et al., 2011), ovarian cancer (Curiel et al., 2003), hepatocellular carcinoma (Wu et al., 2009) and prostate cancer (Sfanos et al., 2009). CD4^{+} tumour-infiltrating lymphocytes (TILs) also showed high expression of PD-1 associated with impaired effector functions. Moreover, PD-1 expression has shown prognostic value in breast cancer (Ahmadzadeh et al., 2009; Ghebeh et al., 2008; Gu-Trantien et al., 2013). Interestingly, tumour-infiltrating T cells exhibited persistently up-regulated expression of the activator protein 1 (AP-1) subunit c-Fos during tumour progression. This unexpected immunosuppressive effect of c-Fos was mediated through the induced expression of PD-1 via the direct binding of c-Fos to the AP-1-binding site in the Pdcd1 (gene encoding PD-1) promoter (Xiao et al., 2012a). Because many tumours are highly infiltrated with Treg cells, PD-1 blockade may also enhance anti-tumour immune responses by diminishing their number and/or suppressive activity (Pardoll, 2012). PD-L1 blockade, however, has been shown to improve myeloid dendritic cell-mediated (MDC) anti-tumour immunity through enhanced MDC-mediated T cell activation, accompanied by downregulation of T-cell IL-10 and upregulation of IL-2 and IFN-γ (Curiel et al., 2003).

2.3. *Clinical trials*

There are currently 101 clinical trials investigating PD-1 and PD-L1 blocking clinical efficacy in a variety of cancers. First evaluated in large phase 1 studies, nivolumab (BMS-936558) and pembrolizumab (MK-3475), humanised-monoclonal immunoglobulin G4 (IgG4) antibodies to PD-1, demonstrated durable response rates with acceptable toxicity in patients with advanced melanoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma or Hodgkin lymphoma (Ansell et al., 2015; Hamid et al., 2013; Topalian et al., 2014). Nivolumab and pembrolizumab are now both approved by the FDA for the treatment of patients with melanoma who have received ipilimumab and, if relevant, a BRAF inhibitor. Recently, nivolumab demonstrated a higher rate of objective response than dacarbazine in a phase III study in patients with ipilimumab-refractory metastatic melanoma, thus improving the overall survival of patients treated with immunotherapy compared to chemotherapy (Robert et al., 2015) (Table 2). Nivolumab is now being evaluated in a large phase III trial in patients with other solid tumours, such as non–small cell lung cancer and renal cell carcinoma (Topalian et al., 2012). Although most of the clinical trials have investigated PD-1 and PD-L1 clinical efficacy in patients with solid tumours, anti–PD-1 therapies have also demonstrated promising outcomes in patients with haematologic malignancies, such as Hodgkin Lymphoma (Ansell et al., 2015; Berger et al., 2008; Lesokhin et al., 2015). BMS-936559, an anti-PDL1 humanised IgG4 mAb was evaluated in a total of 207 patients with advanced cancers, including NSCLC, melanoma, and renal-cell cancer. It induced durable tumour regression at 24 weeks in a number of these patients (6–17%) (Brahmer et al., 2012).

2.4. *TIGIT*

The co-inhibitory molecule TIGIT (T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif [ITIM] domain, also known as Vstm3, Vsig9 or WUCAM) is a protein of the IgSF and consists of two ITIMs, a transmembrane domain and an immunoglobulin variable (IgV) domain (Levin et al., 2011; Yu et al., 2009). TIGIT is partially conserved between species and has 58% homology between mouse and human amino acid sequences (Yu et al., 2009). TIGIT expression patterns are also similar between both species: it is expressed on various T cell subsets such as resting and activated Tregs, activated CD8^{+} and CD4^{+} effectors and particularly on memory T cells (Boles et al., 2009; Levin et al., 2011; Stanietsky et al., 2009; Yu et al., 2009). Furthermore, TIGIT expression is associated with other exhaustion markers such as PD-1 and TIM-3 on murine melanoma specific CD4^{+} T effector and Treg cells during tumour recurrence (Goding et al., 2013). Similarly, Johnston and colleagues recently demonstrated that high TIGIT expression strongly correlated with PD-1 expression on exhausted CD8^{+} and CD4^{+} T cells but could not be detected on non-haematopoietic, myeloid or tumour cells (Johnston et al., 2014) (Table 1).

TIGIT binds with high affinity to Ig-like molecules called nectins, such as CD155 (poliovirus receptor (PVR), NECL5) and CD112 (PVRL2, NECTIN-2) and with lower affinity to CD113 (PVRL3, NECTIN-3) (Yu et al., 2009). TIGIT outcompetes CD226 and CD96 for the binding of CD155 due to its higher binding affinity for the two ligands (Clark et al., 2009; Stanietsky et al., 2013; Yu et al., 2009). CD155, CD112 and

CD113 demonstrate similar expression patterns: they are expressed on fibroblasts, endothelial and epithelial cells, on dendritic cells (DCs) and also on many human tumours (Carlsten et al., 2007, 2009; El-Sherbiny et al., 2007; Castriconi et al., 2004; Fuchs and Colonna, 2006; Iwasaki et al., 2002; Nishiwada et al., 2015; Sakisaka and Takai, 2004; Sloan et al., 2005; Pende et al., 2006, 2005; Donaghy et al., 2009; Lopez et al., 1998; Martin et al., 2013; Maniwa et al., 2012). The third TIGIT ligand CD113 is, however, the only Nectin molecule expressed on T cells (Devilard et al., 2013).

There are two separate T cell inhibitory mechanisms mediated by TIGIT: one T cell intrinsic and the other dependent on interaction with its ligand on APCs (Joller et al., 2011; Yu et al., 2009). Engagement of TIGIT by one of its ligands or an agonistic antibody results in the inhibition of $CD4^+$ T cell proliferation and pro-inflammatory cytokine production *in vitro* and *in vivo* (Joller et al., 2011; Levin et al., 2011; Yu et al., 2009). TIGIT can additionally directly inhibit cytotoxic activity of human and murine NK cells (Li et al., 2014; Stanietsky et al., 2009). In human peripheral blood, interaction of TIGIT with CD155 on monocyte derived DCs has been shown to increase their IL-10 and decrease IL-12, IL-6 and IL-18 secretion (Yu et al., 2009). T cell intrinsic function of mouse and human TIGIT was demonstrated by the use of agonistic antibodies that can directly suppress T cell proliferation and production of pro-inflammatory cytokines in the absence of APCs (Joller et al., 2011; Levin et al., 2011). In humans, engagement of TIGIT with an agonistic mAb inhibited the expression of early activation markers CD25 and CD69 and the production of IL-2 and IFN-γ (Levin et al., 2011). In mice, it was shown on transcriptional level that TIGIT suppresses T cell responses via down-regulation of the expression of the TCR alpha chain and TCR components while simultaneously up-regulating the expression of the anti-apoptotic molecule BCL-XL and cytokine receptors important for T cell maintenance such as IL-2R, IL-7R and IL-15R (Joller et al., 2011; Levin et al., 2011; Lozano et al., 2012). TIGIT is also capable of inhibiting primary human and murine $CD8^+$ T cell function by preventing the homodimerisation of the costimulatory receptor CD226 in *cis* and thereby impairing its function (Johnston et al., 2014). An anti-TIGIT blocking antibody disrupts the TIGIT:CD226 interaction and restores CD226 homodimerisation and thus its co-stimulatory function (Grogan et al., 2015).

There are distinct similarities between the CTLA-4/CD28 axis and the relationship between TIGIT and CD226: TIGIT is upregulated upon T cell activation and is able to outcompete the activating receptor CD226. TIGIT might therefore contribute to suppression of immune responses and autoimmunity in a similar fashion as CTLA-4. Chan and colleagues suggest the use of a TIGIT blocking antibody for immunotherapy for cancers in which T cell, CD155 and CD226 play an important role in controlling the tumour. It is not clear, however, if a blocking TIGIT mAb would be beneficial in the context of an NK cell-controlled tumours (Chan et al., 2012). Although TIGIT was shown to be widely expressed on murine Tregs (Levin et al., 2011; Yu et al., 2009), recent reports illustrated that a specific Th1- and Th17- (but not Th2) response suppressing a subset of activated Tregs displayed significantly increased TIGIT expression (Joller et al., 2014). Upon TIGIT engagement, this Treg subset showed increased production of the anti-inflammatory cytokine IL-10, which specifically inhibits Th1 and promotes Th2 responses (Chan et al., 2003; Joller et al., 2014). An increased Th2 cytokine production was also observed when human T cells were cultured with $TIGIT^+$ Tregs *in vitro* (Joller et al., 2014). Interestingly, TIGIT was also found to be expressed on another human Treg subset, $Helios^+$ memory Tregs (Bin Dhuban et al., 2015). Furthermore, the TIGIT locus was found to be hypomethylated in activated human Tregs, facilitating increased TIGIT expression. It was therefore suggested that Foxp3 binding and demethylation can coordinate TIGIT expression in this T cell subset (Zhang et al., 2013).

Transgenic mice expressing TIGIT on T and B cells and TIGIT deficient mice do not develop any spontaneous autoimmune disorders (Joller et al., 2011; Levin et al., 2011). However, T cells from TIGIT KO mice produce more IL-6, IFN-γ and IL-17 upon activation in comparison to wild-type (wt) cells in model of induced autoimmune encephalitis (Burton et al., 2014; Joller et al., 2011). These results suggest that TIGIT limits autoimmunity and contributes to the development and maintenance of peripheral tolerance but is not indispensable in steady state immunity. It was recently demonstrated in a murine model of chronic LCMV infection that TIGIT was crucial in the regulation of inflammatory cytokine (IFN-γ and TNFα) production of exhausted or chronically stimulated $CD8^+$ and $CD4^+$ T cells (Grogan et al., 2015). Grogan and colleagues further suggest that although there is a synergy between the inhibitory activity of PD-1 and TIGIT, the latter does not exert an extensive inhibitory effect as PD-1 but has a specific role of suppressing T cell effector function and cytokine production (Grogan et al., 2015). Several reports demonstrate that tumour infiltrating $CD8^+$ T cells often co-express TIGIT with PD-1 (Chauvin et al., 2015; Johnston et al., 2014).

TIGIT is highly expressed at the RNA level in solid tumours such as breast carcinoma, colon adenocarcinoma, renal clear cell carcinoma, uterine corpus endometrioid carcinoma and expression correlates with $CD8^+$ T cell infiltration of these tumours (Cancer Genome Atlas, 2012a, b; Cancer Genome Atlas Research, 2012, 2013; Johnston et al., 2014). These results were confirmed at protein level in murine models (mammary carcinoma EMT6 and colon carcinoma CT26) and tumour samples from patients with NSCLC or colorectal carcinoma. Johnston and colleagues further demonstrated that simultaneous blockade of TIGIT and PDL-1 resulted in rejection of CT26 tumours and that 70% of the treated mice displayed long term complete tumour eradication (Johnston et al., 2014). $CD8^+$ but not $CD4^+$ tumour reactive T cells from anti-TIGIT and anti-PD-L1 treated mice showed increased production of both TNFα and IFN-γ. Interestingly, the effect of anti-TIGIT and anti-PD-L1 treatment could be partially reversed by co-injecting anti-CD226, suggesting that TIGIT suppression of $CD8^+$ T cell responses is dependent on CD226 (Johnston et al., 2014). Combination of TIGIT and PD-L1 blocking antibodies also promoted complete remission of both MC38 (fibrosarcoma) and EMT6 tumours in *in vivo* (Grogan et al., 2015).

There are currently two patents published which support the role of modulation of TIGIT signalling in immunotherapy. Genentech's first patent suggests and outlines the suitability of blocking TIGIT in the treatment of immune related diseases such as psoriasis, arthritis, inflammatory bowel disease and

breast cancer, and using aberrant TIGIT expression or activity as diagnostic tool for these diseases (Clark et al., 2009). The most recent patent by Genentech describes the efficacy of a combination treatment with an anti-PD-1 or anti-PD-L1 antibody (MPDL3280A) and an inhibitor for TIGIT expression/activity to treat or delay the progression of cancer or and immune related disease. The agents used to modulate TIGIT expression and/or activity were an inhibitory mAb, a Fab fragment, an aptamer, a small molecule inhibitor, an inhibitory nucleic acid or polypeptide (Grogan et al., 2015). Collectively, these data indicate that TIGIT shows great promise as one of the next targets in trials for immunotherapy in, for example, metastatic melanoma and breast cancer.

2.5. *VISTA/PD-1H (V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation)*

VISTA (also called PD-1H, Dies1 or Gi24) is a type I transmembrane protein with a single extracellular IgV domain containing three additional cysteine residues that are not present in other Ig superfamily members (Wang et al., 2011). Human and murine VISTA share 90% homology and are constitutively expressed on haematopoietic cell subsets, including T cells, macrophages and dendritic cells (Lines et al., 2014a). VISTA is present at a greater density on $CD11b^{high}$ myeloid cells than on $CD4^+$, $CD8^+$ T cells and Tregs (Flies et al., 2011; Le Mercier et al., 2014) (Table 1). While the extracellular domain of VISTA shares significant sequence homology (24%) with the B7 family ligand PD-L1, it displays a distinct expression pattern. VISTA is not found on B cells, NK cells or non-hematopoietic tumour cells (Flies et al., 2011; Le Mercier et al., 2014; Wang et al., 2011). First identified as an inhibitory ligand on APCs, suppressing $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production, VISTA is now also considered as an inhibitory receptor on T cells (Flies et al., 2014; Wang et al., 2011). However, the binding partner(s) of VISTA responsible for the potential reverse signalling between T cells and APCs remains unknown (Lines et al., 2014b). Both naïve and antigen-stimulated cells are sensitive to VISTA-induced suppression, suggesting a constitutive expression on resting T cells (Lines et al., 2014b; Wang et al., 2011). Administration of an antagonistic VISTA mAb was shown to enhance proliferation and effector molecule production (i.e. IFN-γ and granzyme B) in $CD8^+$ T cells (Le Mercier et al., 2014). Similarly, experiments using VISTA–KO mice indicate that VISTA expressed on $CD4^+$ T cells limits both their activation and function (Wang et al., 2011). Human and murine VISTA appear to have indistinguishable functional properties. First, VISTA was found to induce a durable suppression of human $CD4^+$ and $CD8^+$ T cell proliferation and to reduce the production of IL-10, TNFα (both $CD4^+$ and $CD8^+$ T cells) and IFN-γ ($CD4^+$ T cells only) (Le Mercier et al., 2014; Lines et al., 2014a). Additionally, VISTA-Ig fusion protein was effective at suppression of memory ($CD45RO^+$) and effector ($CD27^-$) $CD4^+$ T cell subsets (Lines et al., 2014b). Finally, under conditions promoting T cell proliferation, an enhanced conversion of murine naïve T cells into $Foxp3^+$ T cells was observed *in vitro*, an effect also seen on human $CD4^+$ T cells treated with human-VISTA-Ig (Lines et al., 2014a).

Regarding VISTA function *in vivo*, depletion of $CD4^+$ T cells in a GL261 brain tumour model has been shown to suppress the radiotherapy-induced antitumour effect in VISTA–KO mice whereas depletion of $CD8^+$ T cells had no impact on tumour growth or on the overall survival (Flies et al., 2014). Importantly, both human and murine VISTA are predominantly expressed on tumour-infiltrating leukocytes such as myeloid derived suppressor cells, tumour associated macrophages and dendritic cells (Lines et al., 2014a). VISTA blockade enhances antitumour immunity in multiple tumour models as shown by suppression or delay of tumour growth of the MB49 bladder cancer cells, the B16-OVA melanoma and the poorly-immunogenic B16-BL6 melanoma (Le Mercier et al., 2014). A reduction of tumour-infiltrating monocytic myeloid-derived suppressor cells ($CD11b^+Gr1^+CD11C^-$ MDSCs) was observed as well as an increase of tumour-infiltrating $CD4^+$ and $CD8^+$ T cells.

Thus, VISTA's immunosuppressive and immunoregulatory functions, as well as its consistent expression on leukocytes within tumours make it a relevant target with a broad spectrum of clinical applications. As with PD-L1, it would be interesting to assess VISTA expression on haematological cancer cells. Indeed, PD-L1 overexpression, reported in multiple myeloma, Hodgkin Lymphoma or AML was associated with invasiveness and tumour cell resistance to T lymphocyte cytotoxicity (Iwai et al., 2002; Yamamoto et al., 2008). A clinical trial conducted with a PD-L1 blocking antibody in patients with various haematologic malignancies also demonstrated some evidence of clinical activity (Berger et al., 2008). As VISTA displays significant homology with PD-L1 and its expression is limited to hematopoietic cell subsets, a blocking antibody could also be an effective therapeutic strategy. Importantly, treatment with VISTA-specific mAb has already shown a therapeutic value in manipulating immune responses, as a strategy to prevent the induction of GVHD (Flies et al., 2011).

Although VISTA has been observed within the tumour microenvironment (TME) in human tumours, a comprehensive study of the correlation of VISTA expression with patient outcome in different tumour types is warranted. Antibodies targeting VISTA for cancer immunotherapy are already under development by Johnson & Johnson and VISTA may soon be a part of the immunotherapy revolution (Lines et al., 2014b). Moreover, VISTA, PD1, and CTLA4 appear to induce distinct signalling pathways rendering the multiple blockade agents potentially synergistic.

2.6. *LAG-3*

Human LAG-3 (CD223, lymphocyte activation gene 3) is a type I membrane protein belonging to the IgSF. LAG-3 forms four extracellular IgSF domains and its structure resembles the CD4 molecule. Genes encoding both proteins share 20% homology and show similar exon/intron organisation and chromosomal location, suggesting that both proteins are closely related (Triebel et al., 1990). Murine LAG-3 also resembles CD4 in structure and sequence (Miyazaki et al., 1996; Workman et al., 2002).

Initially, it was shown that LAG-3 was expressed by activated T and NK cells (Triebel et al., 1990). More detailed analysis demonstrated that mouse LAG-3 is expressed by a small

fraction of memory like T cells, 20% of γδ T cells and around 10% of NK cells. Both $CD4^+$ and $CD8^+$ T cells upregulate LAG-3 expression upon activation *in vitro* (Workman et al., 2002). LAG-3 is particularly highly expressed by activated regulatory T cells, although this was demonstrated at the mRNA rather than at the protein level (Huang et al., 2004). Exhausted $CD8^+$ T cells in an LCMV infection model were also shown to express LAG-3 highly along with PD-1 (Wherry et al., 2007) (Table 1).

As suggested by the similarity to CD4, the MHC-II molecule was considered an obvious candidate for a LAG-3 ligand. Indeed, both human and mouse LAG-3 were shown to interact with MHC-II molecules with even higher affinity than CD4 (Baixeras et al., 1992; Workman et al., 2002). These discoveries suggested that LAG-3 might be involved in regulation of T-cell mediated immune responses. Results of the first *in vitro* studies showed that LAG-3 mediates an inhibitory signal to T cells as blocking of LAG-3 by a specific antibody increased proliferation of human T cells (Huard et al., 1994). Also the ectopic expression of LAG-3 on mouse $CD4^+$ T cells reduced their proliferation (Huang et al., 2004). Relatively high expression of LAG-3 by Treg cells raised the question of possible involvement of this molecule in Treg homoeostasis and function. Indeed, the group of D. Vignalli demonstrated that LAG-3 deficient Treg cells exhibit reduced regulatory activity (Huang et al., 2004). However, LAG-3 KO mice do not develop spontaneous lymphoproliferative disease, in contrast to CTLA-4 KO or CTLA-4 Treg specific KO mice (Kajsa, 2008; Miyazaki et al., 1996; Workman and Vignali, 2005). In older LAG-3 deficient animals, a cells-intrinsic, Treg independent expansion of both $CD4^+$ and $CD8^+$ T cells was observed (Workman and Vignali, 2005).

Mechanistic studies on LAG-3 function showed that it associates with TCR/CD3 complexes on T cells and LAG-3 cross-linking inhibited the calcium response to CD3 stimulation (Hannier et al., 1998; Hannier and Triebel, 1999) but the downstream signalling from LAG-3 remains largely unknown. It was established that the regulation of T cell homoeostasis by LAG-3 requires the conserved KIEELE motif in the cytoplasmic tail of the protein (Workman and Vignali, 2005). In 2001, the LAG-3-associated protein (LAP) was identified in activated human T cells and shown to bind to the Glu-Pro (EP) repeated motifs in the intracellular domain of LAG-3 (Iouzalen et al., 2001). Further binding partners of either LAP or LAG-3 have not been discovered so far.

LAG-3 appears to have a more complex role in immune homoeostasis than just inhibiting T cell activation. Binding of LAG-3 to MHC-II positive monocytes or dendritic cells leads to their activation and an increase of chemokine production (MDC, CCL22, TARC, CCL17) (Avice et al., 1999; Annunziato et al., 1996). LAG-3 also induces the upregulation of the cell surface receptors CD40, CD80 and CD86 and the expression of the maturation marker CD83 on monocyte-derived DCs. Furthermore, it was demonstrated that DCs activation mediated by MHC-II engagement of LAG-3 occurs through the kinase p72syk (Andreae et al., 2003). Interestingly, MHC-II can be also expressed by certain types of tumour cells. Interaction of LAG-3 with MHC II expressed on melanoma cells upregulates both MAPK/Erk and PI3K/Akt pathways, albeit with different kinetics. Inhibition studies using specific inhibitors of both pathways provided evidence of their involvement in the LAG-3–induced protection from apoptosis (Hemon et al., 2011). The engagement of LAG-3 with MHC-II on tumour cells may therefore provide a survival signal to them.

In addition to the transmembrane protein, soluble LAG-3 (sLAG-3) formed by an alternatively spliced RNA can be detected in the serum of healthy individuals (Annunziato et al., 1996). Soluble LAG-3 was shown to bind MHC-II and activate APCs but it is not clear if sLAG-3 has any direct impact on T cells. One could assume that binding of sLAG-3 to MHC-II blocks interaction of LAG-3 with MHC-II thus indirectly preventing T cell inhibition. The first attempts of using LAG-3 in immunotherapies focused on the adjuvant function of the soluble form of LAG-3 rather than on blocking its signalling to T cells. Multiple studies with sLAG-3-Ig (soluble LAG-3 fused to human Ig) were performed with different mouse tumour models. Co-administration of LAG-3-Ig with irradiated tumour cells [MCA205 (murine sarcoma), TS/A (mammary adenocarcinoma) and RENCA – (renal adenocarcinoma)] reduced the growth of primary tumours (Prigent et al., 1999). Addition of LAG-3 to cultured human $CD8^+$ T cells isolated from PBMCs from patients with malignancies increased the expansion of antigen specific T cells *in vitro* (Casati et al., 2006). Furthermore, soluble LAG-3 was found to be a good prognostic factor in human breast cancer expressing oestrogen or progesterone receptors. High pre-treatment serum level of sLAG-3 serves as a marker for favourable prognosis for patients with this type of malignancy (Triebel et al., 2006).

Immutep (IMP321, soluble LAG-3-Ig fusion protein) entered clinical trials involving patients with advanced malignancies. Phase I trial with patients with advanced renal cell carcinoma showed an increase in numbers of activated $CD8^+$ T cells in the group treated with IMP321 (Brignone et al., 2009). IMP321 was also tested in combination with chemotherapies or other immune therapies: sLAG-3-Ig together with paclitaxel was used in patients with metastatic breast carcinoma, and after a few months of application the group treated with IMP321 showed increased numbers of activated APCs and cytotoxic NK and $CD8^+$ T cells (Brignone et al., 2010). The encouraging results from the phase I clinical trials supported further combined therapies with Immutep.

In parallel, antibody-based therapies focusing on blocking the interaction between T cell-expressed LAG-3 with MHC-II molecule expressed by APCs or tumour cells were developed. It was shown that TILs, especially $CD8^+$ T cells, infiltrating murine renal cell carcinoma and melanoma express LAG-3 (Demeure et al., 2001). Blocking of LAG-3 with a specific antibody or genetic ablation of LAG-3 in a mouse model of prostate cancer (ProHA × TRAMP) resulted in an accumulation of antigen specific, activated $CD8^+$ T cells and the observed effect was independent of the presence of $CD4^+$ T cells (Grosso et al., 2007). The impact of LAG-3 on $CD8^+$ T cells has been somewhat puzzling, as these cells do not bind to MHC-II molecules. One possible explanation for these results would be the existence of an alternative ligand. Recently it was reported that LAG-3 also binds Galectin-3, a 31 kDa lectin. Depletion of *Galectin-3* resulted in an expansion of $CD8^+$ T cells in the NT2.5 mammary tumour. In addition, galectin-3 mediated suppression on IFN-γ production by T cells was shown to be dependent on the interaction with LAG-3 but not with PD-1 (Kouo et al., 2015).

A set of human LAG-3 binding antibodies was patented first by Medarex and the optimised version of the antibodies by Bristol-Myers Squibb. A phase I clinical trial is currently testing single agent therapy with BMS-986016 (anti-LAG-3 antibody, Bristol-Myers Squibb, source: Clinicaltrial.gov) in patients with relapsed or refractory chronic lymphocytic leukaemia, lymphomas and multiple myeloma. The same agent is being evaluated in combination with nivolumab (anti-PD1) in advanced solid tumours (Clinicaltrial.gov). LAG-3, with its high expression on regulatory and activated T cells, has a great potential to be an important target for antibody-based cancer immunotherapies.

2.7. *TIMs (T cell Ig and mucin domain)*

In mice, the TIM gene family is represented by four members (TIM-1–TIM-4) and four additional predicted genes (TIM-5–TIM-8) clustering on chromosome 11B1.1. In human, only three members (TIMs 1, 3 and 4) have been identified so far and they are expressed on chromosome 5q33.2. (Meyers et al., 2005b). The TIM molecules are type I transmembrane proteins, containing a single IgV domain followed by a variable length mucin domain and cytoplasmic tail with tyrosine-based signalling motif, except for TIM-4 (Chattopadhyay et al., 2009).

TIM-1/TIM-2 and TIM-3 are expressed on T cells, while TIM-4 is primarily expressed on antigen-presenting cells (Rodriguez-Manzanet et al., 2009). TIM-1 is expressed on $CD4^+$ T cells after activation and its expression is preferentially sustained in Th2 cells (Meyers et al., 2005a) (Table 1). TIM-4 is the ligand for TIM-1 and both are known to deliver co-stimulating signals to T cells. The administration of soluble TIM-1–Ig and TIM-4–Ig *in vivo* induces hyperproliferation of T cells (Meyers et al., 2005a) and the use of agonist antibody to TIM-1 upregulates the production of IL-4 and IFN-γ in unpolarised T cells (Umetsu et al., 2005). Nevertheless, an inhibitory potential for both TIM-1 and TIM-4 has been reported. They have been shown to inhibit the Th1 and Th17 responses of $CD4^+$ T cells (Mizui et al., 2008) through a still unknown mechanism but independently of each other (Cao et al., 2011; Xiao et al., 2011). Interestingly, TIM-1 seems to play a critical role in maintaining the suppressive function of regulatory B cells (Bregs) as shown by impaired IL-10 production in B cells, associated with autoimmune disease in Tim-1Δmucin mice (Xiao et al., 2012b). Of note, TIM-1, TIM-3 and TIM-4 are phosphatidylserine (PtdSer) receptors involved in the engulfment of apoptotic cells and intercellular signalling with exosomes (DeKruyff et al., 2010; Nakayama et al., 2009). Yet, the importance of such interactions in the regulation of T cell responses as well as the TIM-1/TIM4 inhibitory activity remain unclear.

It is now well established that TIM-3 functions as a negative immune-checkpoint molecule. This activation-induced inhibitory molecule is involved in tolerance and induces T-cell exhaustion in chronic viral infection and cancers (Jones et al., 2008; Sabatos et al., 2003; Sanchez-Fueyo et al., 2003). TIM-3 is selectively expressed on a subset of murine IFNγ-secreting Th1 $CD4^+$ and $CD8^+$ T cells but not Th2 cells (Monney et al., 2002). In human, it is also expressed on a subset of activated $CD4^+$, $CD8^+$ and at lower levels on Th17 cells (Chen et al., 2006; Hastings et al., 2009; Nakae et al., 2007). *Ex vivo* stimulation indicates that Tim-3 is more easily induced on $CD8^+$ than on $CD4^+$ T cells (Jones et al., 2008; Mujib et al., 2012). Furthermore, TIM-3 expression can also be upregulated independently of TCR or antigenic stimulation in an inflammatory environment rich in cytokines (IL-2, IL-7, IL-15, IL-21), an effect that is blocked by a γc cytokine receptor-neutralising antibody (Mujib et al., 2012) (Table 1). Additionally, TIM-3 proteins can be produced in a soluble form or can be shed from the cell surface, cleaved by membrane-associated proteases (Geng et al., 2006; Moller-Hackbarth et al., 2013). As a cell-free ligand, sTIM-3 can still significantly impair T cell anti-tumour immunity, as shown by decreased anti-tumour CTL activity and reduced amount of tumour-infiltrating lymphocytes (Geng et al., 2006).

In addition to the interaction with PtdSer, the IgV domain of TIM-3 can bind to the alarmin protein HMGB1 (High-Mobility Group Box 1) and suppress the activation of dendritic cells associated with tumours. TIM-3 and HMGB1 interaction blocks the trafficking of nucleic acids into endosomes, thus decreasing toll like receptor stimulation (Chiba et al., 2012; Gorman and Colgan, 2014). Whether HMGB1 is being sequestered by TIM-3-expressing T cells or if such contact regulates T cell responses remains unknown.

A study from Zhu et al. was the first to demonstrate that galectin-9, a soluble protein with specificity for carbohydrate chains, can be a ligand for TIM-3, impacting T cells responses. TIM-3 can indeed bind to galectin-9 via its two carbohydrate recognition domains. This interaction has been shown to be critical for inducing cell death of antigen specific $CD4^+$ (Zhu et al., 2005) and $CD8^+$ T cells (Sehrawat et al., 2010), a process that is reduced by the ligation of Bat3 (leucocyte antigen B-associated transcript 3; also known as BAG6) to the intracellular tail of TIM-3 (Rangachari et al., 2012; Sabatos et al., 2003; Saresella et al., 2014). Even though galectin-9 can mediate a calcium–calpain–caspase-1 dependent apoptosis of Th1 cells (Kashio et al., 2003), the cell death is not completely abolished in TIM-3-deficient cells, suggesting that an additional receptor is possibly involved in this mechanism (Zhu et al., 2005). As galectins can induce both necrosis and apoptosis, the Fas–Fas ligand pathway has been considered as the supplementary target (Vercammen et al., 1998). A subsequent study demonstrated that galectin-9 can induce cytokine secretion and apoptosis of Th1 and Th2 T cells independently of TIM-3 (Su et al., 2011). A further report, however, provided evidence refuting a role for interactions between galectin-9 and TIM-3 in regulating T cell responses (Leitner et al., 2013). Finally, another mechanism explaining the TIM-3/galectin-9 mediated indirect inhibition of Th1 immune responses is the expansion of MDSCs resulting from TIM-3 overexpression on T cells (Dardalhon et al., 2010; Sabatos et al., 2003). Galectin-9 expression is widely distributed in tissues involved in the immune system and was first identified as a tumour antigen in Hodgkin lymphoma (Tureci et al., 1997). Interestingly, galectin-9 levels vary when comparing normal and tumour tissue. A low or decreased expression has been reported in several solid tumours including breast, lung, melanoma, renal, adrenal and prostate tumour cells (Irie et al., 2005) while a high or increased galectin-9 expression has been noted in leukaemia and colon cancer cell lines (Lahm et al., 2001). Altogether, most data demonstrate an inverse

relation between galectin-9 expression and cancer progression for the majority of tumours (Heusschen et al., 2013; Jiang et al., 2013). Expression of galectin-9 expression and its splice variant remain to be characterised in the tumour microenvironment, and whether the loss of galectin-9 allows tumours to escape from immune control needs to be investigated.

It has been shown that the TIM-3 IgV domain interacts with at least one other ligand, expressed on $CD4^+$, $CD8^+$, regulatory T cells, B cells, macrophages, and dendritic cells (Cao et al., 2007). Recently, Huang et al., demonstrated that TIM-3 co-localises with the carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) (Huang et al., 2015), expressed on activated $CD4^+$ and $CD8^+$ T cells and known to be involved in T-cell inhibition (Boulton and Gray-Owen, 2002; Iijima et al., 2004; Nagaishi et al., 2006). Interestingly, Th1- polarised cells from TIM-3-overexpressing *Ceacam1*$^{-/-}$ mice were resistant to galectin-9-induced apoptosis, suggesting a distinct role for these two ligands. However, the authors reported that the CEACAM1 and TIM-3 heterodimeric complex is the major factor responsible for T-cell inhibition and human leucocyte antigen-B-associated transcript 3 (BAT3) release. Importantly, TIM-3 expression appears to be regulated by CEACAM1 since *Ceacam1*$^{-/-}$ $CD4^+$ T cells lack TIM-3 expression after activation. The double-positive ($CEACAM1^+$ $TIM\text{-}3^+$) phenotype is essential for both $CD4^+$ T cells and $CD8^+$ T cells to exhibit the tolerance-inducing function of TIM-3 (Huang et al., 2015). Both CEACAM1-L and TIM-3 can recruit SHP-1/SHP-2 phosphatases, respectively, in an ITIM-dependent manner and Lck, CD45, CD148 at the immune synapse to suppress T cell receptor (TCR) signalling (Izzi et al., 1999; Clayton et al., 2014). Downstream, TIM-3 expression is regulated by the Th1 transcriptional protein T-bet (Anderson et al., 2010). As T-bet does not regulate the Th17 differentiation (Park et al., 2005), other transcription factors might be involved in the regulation of Tim-3 expression in Th17 cells.

Anti-tumour immunity is hindered by T cells with an exhausted phenotype (Fridman et al., 2012) and TIM-3 has been reported to be co-expressed with PD-1 on tumour-specific $CD8^+$ T cells in mice (Sakuishi et al., 2010) and in patients with advanced melanoma (Baitsch et al., 2012; Fourcade et al., 2010). In both case, $TIM\text{-}3^+$ $PD\text{-}1^+$ TILs represent the most abundant and dysfunctional population of TILs with a severe exhausted phenotype as defined by the failure to proliferate and to produce IL-2, TNF, and IFN-γ. Recently, $PD\text{-}1^+$ $TIM\text{-}3^+$ $CEACAM1^+$ T cells were also characterised by extremely low intracellular IL-2 and TNF-α expression consistent with exhaustion. However, TIM-3 blockade by itself displays a modest therapeutic effect in a murine model of colorectal cancer (CT26) (Sakuishi et al., 2010). CEACAM1 and TIM-3 co-blockade is associated with increased $CD8^+$ and $CD4^+$ TILs, together with enhanced IFN-γ production and decreased IL-10 production. This dual-blockade improved elimination of CT26 tumours (Huang et al., 2015). Finally, dual blockade of TIM-3 and PD-1 pathways can reverse T cell exhaustion and restore anti-tumour immunity with restoration of anti-tumour $CD8^+$ T cell cytokine production and enhanced proliferation (Fourcade et al., 2010; Ngiow et al., 2011; Sakuishi et al., 2010). Similarly, the simultaneous targeting of TIM-3 and PD-1 has been shown to rescue $CD8^+$ T cells from exhaustion in a model of chronic infection (Takamura et al., 2010). To conclude, manipulation of the TIM-3 pathway could be a promising molecular target for therapeutic intervention in autoimmune disease, chronic viral infections and cancer.

2.8. *Ceacam1*

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1, CD66a, BGP) is a member of the IgSF and has been shown to have co-inhibitory function on T cells *in vitro* in both human and murine cells and *in vivo* in a murine model of Th1-mediated colitis (Chen and Shively, 2004; Iijima et al., 2004; Kammerer et al., 1998; Markel et al., 2002b; Nakajima et al., 2002). The human and murine *Ceacam1* genes are highly conserved and can generate 12 and 4 alternative splice variants, respectively. The different splice variants exist as secreted molecules or transmembrane receptors with varying lengths of the cytosolic tail. The different isoforms of the transmembrane molecules are termed long (-L) or short (-S) due to their cytoplasmic domain which contains two ITIM domains in the long isoforms in mouse and human (Beauchemin et al., 1999; Tan et al., 2002). Semiquantitative analysis in T cells suggests that CEACAM1-L isoforms predominate over CEACAM1-S isoforms (Singer et al., 2002). The short isoform CEACAM1-4S provides T cell co-stimulation and can function independently of CEACAM1-L isoforms and their inhibitory signalling (Chen et al., 2004, 2012b, 2004, 2012). Unlike other family members, CEACAM1 is the only one expressed on $CD8^+$ and $CD4^+$ T cells, immediately after activation through the TCR-CD3 complex *ex vivo* (Kammerer et al., 1998; Morales et al., 1999; Nakajima et al., 2002) (Table 1). The long CEACAM1 isoforms negatively regulate T cell function by ablating TCR-CD3 complex signalling through ZAP-70. The inhibition is initiated by CEACAM1-L associating with the CD3 signalling complex which enables the phosphorylation of the CEACAM1-L ITIMs by *src*-related kinase p56*lck*, thus allowing the recruitment of SHP-1 phosphatase. SHP-1 then dephosphorylates CD3-ζ and ZAP70 and thereby abrogates all downstream functions resulting in the down-modulation of the immune response, including the exocytosis of cytotoxic granule proteins (Chen and Shively, 2004; Chen et al., 2008; Huber et al., 1999; Nakajima et al., 2002). CEACAM1 deficient mice did not display any change in the haematopoietic cell compartment, autoimmune disorder development or in spontaneous tumour growth (Leung et al., 2006). However, engagement of CEACAM1-L with mAbs *in vitro* leads to inhibition of murine and human $CD4^+$ and $CD8^+$ T cell cytokines (IL-2, IL-4, IFN-γ) production and proliferation (Boulton and Gray-Owen, 2002; Chen and Shively, 2004; Morales et al., 1999; Nakajima et al., 2002). The overexpression of CEACAM1-L on naive T cells led to the inhibition of *in vitro* differentiation to both Th1 and Th2 cells and to *in vivo* impaired cytokine secretion by these T cells subsets in an adoptive transfer colitis model (Nagaishi et al., 2006). However, Th1 pathways may be more sensitive to CEACAM1-mediated suppression than Th2 pathways, a result that is similar to previous observations with PD-1 and PD-1 ligand interactions (Latchman et al., 2001). The overexpression of CEACAM1-L also inhibited nuclear translocation of T-bet, GATA-3, STAT-4, and STAT-6 which

suggests that CEACAM1 inhibits not only TCR-CD3 signalling in T cells but also likely IL-4 and IL-12 receptor signalling (Nagaishi et al., 2006). The latter is consistent with previous studies showing CEACAM1 inhibition of IL-2R signalling (Chen and Shively, 2004).

CEACAM1 was originally discovered as a colorectal cancer antigen. It has since been demonstrated that the expression of long- and short-tailed CEACAM1 is vastly dysregulated on various cancers (Hinoda et al., 1988; Gaur et al., 2008; Turbide et al., 1997; Wang et al., 2000). CEACAM1-L is highly expressed in gastric carcinoma, melanoma, metastatic colorectal carcinoma, NSCLC, bladder and thyroid cancer and CEACAM1 isoforms are downregulated in breast cancer, endometrial carcinomas and in the early stages of colon, prostate and liver cancer (Bamberger et al., 1998; Busch et al., 2002; Huang et al., 1998; Neumaier et al., 1993; Riethdorf et al., 1997; Rosenberg et al., 1993). Markel and colleagues demonstrated that CEACAM1 expression allows tumour cells to escape destruction by NK and T cells *in vitro* (Markel et al., 2002a, 2006, 2002b). NK and T cells from melanoma patients express CEACAM1 at high levels, which increases their potential sensitivity to CEACAM1-dependent inhibition by tumour cells (Markel et al., 2002a, 2010, 2006). This is consistent with human CEACAM1-L overexpression in Jurkat cells, which is able to inhibit both Th1 and Th2 cytokine secretion in an ITIM-dependent pathway.

In this context, Ortenberg and colleagues successfully developed an anti-CEACAM1 antibody, which counteracts the CEACAM1-mediated inhibition of T cells by other lymphocytes and melanoma cells *in vitro* and *in vivo*. Specifically, administration of the anti-CEACAM mAb in a human-melanoma xenograft murine model resulted in the inhibition of xenograft growth and elimination of malignant cells by T cells (Ortenberg et al., 2012). There are two patents published involving CEACAM1, one detailing the function and efficacy of newly synthesised peptides to modulate CD66 family member activity (Skubitz and Skubitz, 2001) while cCam Biotherapeutics patented an anti-CEACAM1 therapeutic antibody for use in viral infection and cancer therapy (cCam Biotherapeutics LTD et al.). cCAM Biotherapeutics also recently started a phase I clinical trial with their patented anti-CEACAM1 mAb CM-24 (humanised IgG4) for treatment of advanced or recurrent NSCLC, melanoma, bladder, colorectal, gastric and ovarian cancer (cCAM Biotherapeutics Ltd. NCT02346955).

2.9. LAIR-1

LAIR-1 (leucocyte-associated immunoglobulin-like receptor-1, CD305) was first identified as an inhibitory receptor expressed by human peripheral blood leukocytes in studies focusing on NK-cell mediated toxicity. Human LAIR-1, a type 1 membrane protein structurally related to KIRs, belongs to the IgSF. The intracellular part of LAIR-1 contains the amino acid sequences VTYAQL and ITYAAV, which fit the consensus sequences for ITIMs (Meyaard et al., 1997). LAIR-1 mediates negative signalling to cells through binding of SHP-1 and SHP-2 to ITIMs fragments (Meyaard et al., 1997). It can also function independently from SHP-1 and 2 and signal through C-terminal Src kinase (Csk) (Verbrugge et al., 2006). The murine homologue (mLAIR-1) shares 40% sequence identity with human LAIR-1 and also contains 2 ITIM motifs in the cytoplasmic tail that only associate with SHP-2. LAIR2, another protein highly homologues to LAIR-1 has been described in mice (Lebbink et al., 2004).

LAIR-1 is broadly expressed on immune cells, including 70–80% of human $CD4^+$ and $CD8^+$ T cells (PBMCs), nearly all NK cells and monocytes in healthy individuals (Meyaard et al., 1997). It is expressed at higher levels on human $CD8^+$ than $CD4^+$ T cells. It is also reported that naïve human T cells have higher expression of LAIR-1 than effector or memory cells (Maasho et al., 2005). Expression of LAIR-1 on murine cells resembles the pattern shown in humans but it is not expressed on B cells in contrast to human cells (van der Vuurst de Vries et al., 1999). LAIR-1 expression was recently shown to predict time-to-first-treatment in patients presenting with chronic lymphocytic leukaemia (CLL) (Perbellini et al., 2014). In contrast to the human protein, mLAIR-1 expression is higher on mouse memory than naïve T cells (Tang et al., 2012) (Table 1).

Engagement of the LAIR-1 receptor by a specific antibody caused a decrease in human NK and T cells cytotoxicity, although the antibody did not inhibit T cell proliferation and activation (Meyaard et al., 1999). In contrast, Maasho et al. demonstrated that ligation of LAIR-1 on human $CD8^+$ T cells decreased calcium mobilisation but not $CD8^+$ T cells mediated lysis (Maasho et al., 2005). In 2012, the LAIR-1 knockout mouse was generated and no systemic autoimmune phenotype was observed. The impact on T cells was relatively mild with a slight increase of CD69 and CD44 expression in older animals and increased number of regulatory T cells (Tang et al., 2012).

Both human and murine LAIR-1 bind with high affinity to collagen (Lebbink et al., 2006). In addition, collagens I and III are capable of cross-linking with hLAIR-1. Subsequently it was demonstrated that both soluble and cell surface bound LAIR-1 bind *in vitro* to collagen-expressing cell lines (Rygiel et al., 2011). Collagens are important component of the extracellular matrix (ECM) and various collagens, including collagen I, II, III, V, and IX, show increased deposition during tumour formation which opens a possibility for interventions in cancer immunotherapies.

Currently there are no clinical trials ongoing with antibodies modulating the LAIR-1 pathway. There are several reasons why LAIR-1 might not be considered an optimal target for checkpoint modulating therapies. Firstly, LAIR-1 is highly expressed on naïve human T cells rather than activated ones. Secondly, the results obtained with LAIR-1 KO cells show only slight impact on T cell compartment.

2.10. HVEM-BTLA-CD160 network

HVEM (herpes virus entry mediator) is both, a signalling receptor and ligand for specific inhibitory receptors expressed on T cells. HVEM was first shown to interact with the glycoprotein envelope D (gD) of the Herpes viruses 1 and 2 expressed on T cells and later shown to bind the TNFR family members lymphotoxin-α (LT-α) and lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), as well as to the immunoglobulin superfamily (IgSF)

members BTLA (CD272) and CD160. With a broad expression pattern, including haematopoietic and non-haematopoietic cells, the interaction between these ligand/receptor pairs, either in *cis* (expression on the same cell) or in *trans* (expression on different cells), constitutes a complex co-inhibitory and co-stimulatory network. Thus, HVEM serves as a bidirectional switch for T cell activation, promoting either activation or regulation depending on the ligand engaged and the cellular context.

2.10.1. *HVEM – receptor and ligand*

HVEM is a member of the TNFR family molecules (TNFR14), also called LIGHTR in reference to one of its ligands (Montgomery et al., 1996). The HVEM open reading frame encodes a single transmembrane protein with two perfect and two imperfect TNFR-like cysteine-rich domains (CDR). LIGHT and LT-α interact with the CDR2 and CDR3 domains of HVEM and the Ig superfamily molecules BTLA and CD160 bind to CDR1 and partially to CDR2 (Sarrias et al., 2000; Compaan et al., 2005; Cai and Freeman, 2009). HVEM has a short cytoplasmic tail which does not contain a death domain and shares some similarity with 4–1BB and CD40 (Kwon et al., 1997a), for detailed see review: (del Rio et al., 2010). Instead, there are PXQT and IPEEGD motifs that bind to TRAFs (TNFR associated factors) which activate the transcription factors NF-kB and AP-1 (Hsu et al., 1997).

HVEM is widely expressed on immune cells including T and B lymphocytes, NK cells, DCs and myeloid cells as well as non hematopoietic cells in liver, kidney and lungs (Marsters et al., 1997). It is highly expressed on naïve T cells, downregulated shortly upon activation and returns to its steady state levels a few days after activation *in vitro* (Morel et al., 2000; Sedy et al., 2005) (Tables 1 and 3). This expression profile in response to *in vitro* stimulation has not yet been confirmed in human T cells but was demonstrated in human B cells, binding to LIGHT and in DCs after maturation (Duhen et al., 2004). The exact mechanisms underpinning HVEM downregulation is not completely understood but thought to depend on receptor/ligand interactions as monoclonal antibodies blocking HVEM/LIGHT binding prevent HVEM down-regulation (Morel et al., 2000).

Recent studies have shown that HVEM overexpression and gene polymorphisms can both be associated with malignancies such as colorectal cancer, melanoma, and breast cancer as well as haematopoietical malignancies (Table 3). Whilst its role in tumourigenesis has not been completely understood, recent studies demonstrate that high levels of HVEM expression on tumour cells is associated with unfavourable prognosis in patients with oesophageal squamous cell carcinoma (Migita et al., 2014). High levels of HVEM on these patients tumours' correlated with larger tumour size, lymph node metastasis and lower 5-year survival rate. Of relevance,

Table 3 – Expression of BTLA, HVEM and CD160 on T cells and tumour cells.

Molecule	Ligand	Expression on haematopoetic cells	Expression on malignancies	Gene mutations and its expressions/SNPs	Ref.
HVEM	BTLA, CD160, LIGHT, lymphotoxin-α, herpes simplex virus glycoprotein D	T cells, B cells, NK cells, DCs, myeloid cells; T cells: Higher on naïve T cells, drops shortly after activation and than re-expressed	Melanoma, hepatocellular carcinoma, colorectal cancer; MCL, ALL, CLL Melanoma (primary tumour and metastasis), Oesophageal squamous cell carcinoma, Primary plasma cells (Myeloma, plasma cell leukaemia)	Nonsynonymous substitutions in patients with follicular lymphoma - poor prognosis or no difference	Derre et al., 2010, Costello et al., 2003
BTLA	HVEM	T cells, B cells, NK cells, DCs, myeloid cells T cells: Increased upon activation (Human and Murine), Higher On Th1 than Th2, Higher expression by CD4 than CD8 T cells	CLL, SLL, TA+ CD8 T cells in melanoma, MCL, Marginal zone lympohma, Follicular Lymphoma, Burkitt Lymphoma, Hodgkin's Lymphoma	No data	Watanabe et al., 2003, M'Hidi et al., 2009
CD160	HVEM, MHC-I	NK T cells, NKT cells, T cells, $\gamma\delta$Tcell (transmembrane form only on subpopulation of IL-15 activated human NK cells) T cells: Highest expression on exhausted and activated CD8 cells, low expression by CD4 T cells	CLL, MCL, Hairy Cell leukaemia,	No data	Farren et al., 2011, Maiza et al., 1993

tumours expressing high levels of HVEM were also found to be poorly infiltrated by both $CD4^+$ and $CD8^+$ T cells (Migita et al., 2014). In human colorectal cancer HVEM expression was also inversely correlated with the presence of tumour-infiltrating T cells (Inoue et al., 2015). Similar outcomes were observed in patients suffering from hepatocellular carcinoma where high HVEM expression was linked to lower infiltration of $CD4^+$, $CD8^+$ and $CD45RO^+$ T cells. Moreover, the expression of granzyme B, perforin and interferon-c was also decreased in this group (Hokuto et al., 2015). Although the function of HVEM in other malignancies still remains unknown, the current data does support a role for tumour associated HVEM in the negative control of anti-tumour immunity.

2.10.2. *BTLA (CD272) B and T lymphocyte attenuator*

BTLA (CD272) is an Ig-domain-containing transmembrane glycoprotein (Watanabe et al., 2003) that belongs to the CD28 IgSF. Whilst it was first cloned from murine Th1-polarised $CD4^+$ T cells, highest levels of expression are found on B cells, followed by T cells and antigen presenting cells.

Whereas BTLA is expressed at low levels on murine T cells, BTLA is rapidly upregulated during *in vitro* polarisation of T cells to either Th1 or Th2 with higher expression on the latter compartment (Watanabe et al., 2003). In steady state conditions, BTLA expression on murine Tregs is equivalent to that on naïve cells and it remains low even upon TCR stimulation (Tao et al., 2008). In healthy individuals BTLA is expressed on 90% of human T and B lymphocytes freshly isolated from blood (Otsuki et al., 2006). Human T cells differentiated *in vitro* into either to IFN-γ or IL-4 producing cells have shown the same level of BTLA expression (Otsuki et al., 2006). (Tables 1 and 3).

The relevance of BTLA for T cell function and homoeostasis was demonstrated both *in vitro* and *in vivo*. Cross-linking of BTLA on activated T cells results in decreased proliferation and cytokine production (i.e. IL-2, IL-4, IL-10 and IFN-γ). Interestingly, this effect is stronger in $CD4^+$ than in $CD8^+$ T cells which might be due to the differential expression of BTLA on these two subsets (Krieg et al., 2005; Watanabe et al., 2003; Otsuki et al., 2006). BTLA knockout mice did not develop an overt autoimmune phenotype although BTLA-deficient T cells showed a heightened response to stimulation with anti-CD3 (Watanabe et al., 2003).

Although early studies suggested that BTLA and B7x were binding partners, later studies identified HVEM as the ligand for BTLA, and which remains as the only known binding partner for BTLA (Sedy et al., 2005). Upon the interaction with HVEM BTLA delivers inhibitory signal to T cells mediated most likely by SHP-1 and SHP-2 proteins (Chemnitz et al., 2006). However, when BTLA binds to HVEM in *trans* (expressed by adjacent cell) both molecules signal – HVEM sends stimulatory signal through the NF-kB pathway whilst BTLA delivers inhibitory signals as shown in 293T cells (Cheung et al., 2009). In addition, BTLA was shown to form heterodimeric complexes on the same cell on naïve human and mouse T cells, thus blocking the interaction in *trans* (Cheung et al., 2009).

There are several reports investigating BTLA expression in different malignancies. MART-1 specific $CD8^+$ T cells isolated from melanoma patients' peripheral blood expressed high levels of BTLA and showed decreased IFN-γ production upon crosslinking with HVEM (Derre et al., 2010). Another group characterised BTLA expression by NY-ESO-1 specific $CD8^+$ T cells isolated from melanoma patients' blood and showed that $BTLA^+$ $CD8^+$ T cells (which also expressed high levels of PD-1 and TIM-3) produced lower amounts of IFN-γ and TNF-α (Fourcade et al., 2012). Both studies focused on the expression of co-inhibitory receptors on circulating T cells and there is still a paucity of data on the expression of BTLA by tumour infiltrating lymphocytes.

2.10.3. *CD160*

Human CD160 was initially described on NK cells as the target recognised by the BY55 monoclonal antibody (Anumanthan et al., 1998). CD160, which belongs to the IgSF, exists in two isoforms with either a transmembrane domain or a glycosphingolipid anchor to the cell membrane. Both isoforms contain a single immunoglobulin V (IgV)-like domain with low degree of homology to other killer inhibitory receptors (Agrawal et al., 1999). The glycophosphatidylinositol (GPI)-anchored isoform of CD160 is expressed by human $CD56^{dim}CD16^+$ NK, NKT cells, activated $CD8^+$ T cells, and a small fraction of $CD4^+$ T cells whereas the transmembrane isoform is only found in a small subset of IL-15 activated NK cells (Giustiniani et al., 2009). Although CD160 is not expressed on B cells in physiological conditions, its expression was reported on B cell malignancies including CLL, monoclonal B-cell lymphocytosis and hairy cell leukaemia. Most recently CD160 has been suggested as a marker for minimal residual disease in CLL (Farren et al., 2011, 2015) (Table 3).

Similarly, in mice, CD160 expression is highest on NK and memory $CD8^+$ T cells and express at very low levels on $CD4^+$ T cells (Agrawal et al., 1999; Cai et al., 2008; Le Bouteiller et al., 2002; Maeda et al., 2005). For some time, CD160 was considered a good marker for cytotoxic $CD8^+$ T cells, however, later studies failed to demonstrate co-localisation with cytotoxic molecules (Merino et al., 2007). A more recent publication shows that CD160 is upregulated on murine exhausted $CD8^+$ T cells along with PD-1, LAG-3 and CD244 (Blackburn et al., 2009) (Tables 1 and 3).

CD160 was first shown to bind MHC I and later on, its high affinity interaction with HVEM was demonstrated (Maeda et al., 2005; Cai et al., 2008). Binding of HVEM to CD160 expressed on $CD4^+$ T cell was shown to inhibit their proliferation *in vitro*, thus supporting the co-inhibitory nature of this ligand/pair interaction (Cai et al., 2008).

There are several reports describing the function of CD160 in NK cell development, homoeostasis and function, whereas full understanding of its role on T cells is rather scarce. CD160 knockout mice did not show any abnormalities in NK or NKT cell development, however CD160-deficient mice were more susceptible to early onset of tumour growth. This was demonstrated to be due to decreased IFN-γ production by NK cells rather than impaired cytotoxicity (Tu et al., 2015).

Without detailed understanding and characterisation of the expression pattern of HVEM and its ligands on TILs, myeloid cells and tumour cells, it will be very difficult to predict the results of antibody-mediated therapy. Although in 2013 Medarex patented a set of fully human monoclonal anti-BTLA and anti-HVEM antibodies to date no clinical trial has been reported with their usage.

2.11. CD200/CD200R

CD200 (OX-2) and CD200 receptor (CD200R) are type-1 transmembrane glycoproteins that contain two immunoglobulin superfamily (IgSF) domains and are highly conserved between human and mouse (Wright et al., 2003, 2001). In mouse and human CD200 is robustly expressed by cellular components of the CNS as well as on B cells and activated $CD8^+$ and $CD4^+$ T cells (Barclay et al., 2002; Webb and Barclay, 1984; Wright et al., 2001). CD200 receptor (CD200R) is expressed on granulocytes, monocytes and $CD4^+$ T cells in mice (Preston et al., 1997; Wright et al., 2003, 2000). Similarly, CD200R is mainly expressed in human peripheral blood $CD4^+$ and $CD8^+$ T cells, neutrophils, basophils and monocytes and *in vitro* differentiated DCs (Wright et al., 2003). Interestingly, the cytoplasmic tails of both mouse and human CD200 and CD200R do not contain an ITIM motives. The cytoplasmic region of CD200R, however, features an NPxY motif which contains three free tyrosine residues that are phosphorylated upon interaction of CD200R and CD200. The signalling is mediated via adaptor proteins Dok1 and Dok2 that bind the phosphorylated NPXY motif and recruit RasGAP. As RasGAP inhibits the Ras/MAPK pathways, activation of crucial molecules such as ERK, JNK, and p38 MAPK are inhibited upon engagement of CD200R with its ligand (Wright et al., 2003). There are four different isoforms of CD200R, but only CD200R1 expresses the long cytoplasmic tail with the NPXY motif (Gorczynski et al., 2004; Wright et al., 2003).

Characterisation of CD200 KO mice showed its major role as an inhibitory molecule for myeloid cell with no direct impact on T cells (Gorczynski et al., 1999; Hoek et al., 2000). Misstear and colleagues demonstrated, however, that antigen presenting cells expressing CD200 were able to suppress cognate antigen-specific T cell activity such as IFN-γ production and cytotoxic granule mobilisation in mouse and human (Misstear et al., 2012). Similarly, it was shown that human CD200 expressing B cells inhibited Th1 responses (IL-2 and IFN-γ production) (McWhirter et al., 2006). As in CD200 KO mice, there was also a lack of phenotypic changes in the CD200R1 deficient mouse. When immunologically challenged, however, the importance of CD200:CD200R1 in the suppression of immune responses became apparent: CD200R1 KO mice rejected allografts quickly, despite administration of CD200 Fc (Boudakov et al., 2007).

Recent studies have revealed that CD200 is broadly expressed in a variety of cancer cells. Increased expression of CD200 was detected on B cells of CLL patients and was shown to correlate with poor prognosis in acute myeloid leukaemia (AML) (McWhirter et al., 2006; Tonks et al., 2007). Also a large variety of solid tumours were shown to express high levels of CD200 such as (metastatic) melanoma, head and neck carcinoma, ovarian cancer, testicular cancer, malignant mesothelioma, colon carcinoma, multiple myeloma and renal carcinoma and CD200 expression was found to be associated with tumour progression in the majority of these cancers (Moreaux et al., 2006, 2008; Petermann et al., 2007; Siva et al., 2008).

Kretz-Rommel and colleagues demonstrated that in a murine model of CD200 expressing human Burkitt's lymphoma, blocking of the CD200-CD200R interaction by anti-CD200 mAb administration resulted in almost complete suppression of tumour growth and thus suggested the use of anti-CD200 antibodies for the clinic (Kretz-Rommel et al., 2008, 2007). In a mouse model of breast cancer, EMT6 cells increased CD200 expression dramatically only when transplanted into immunocompetent mice. Administration of neutralising anti-CD200 mAbs improved the cytotoxic immune response and led to decreased tumour growth. Interestingly, a strong correlation was found between the levels of a soluble form of CD200 in the serum of EMT6 or EL4 (thymoma) tumour bearing mice and their respective tumour growth, which allowed a non-invasive monitoring of the tumour burden (Gorczynski et al., 2010).

Trillium Therapeutics Inc. holds two patents disclosing different methods of inducing and preventing immune suppression by engaging CD200 or inhibiting CD200:CD200R interaction in the context of cancer, autoimmune diseases, graft rejection and allergies (patent: Gorczynski and Clark, 2005). Furthermore, an assay to detect high levels of soluble CD200 in the serum of cancer patients to diagnose and monitor the disease was established (patent: Gorczynski and Wong, 2009). In addition, Merck & Co (Schering Corp) has published three patents outlining the use of an agonist/antagonist anti-CD200R/anti-CD200 mAb/antibody fragment to induce CD200-mediated T cell inhibition for the treatment inflammatory and autoimmune disorders (Presta, Cherwinski, and Phillips, 2008; Truitt, Rosenblum, and Olasz, 2005). Alexion Pharmaceuticals currently holds three patents concerning CD200 and its modulation for therapy of cancer or autoimmune disorders. Patented are also antagonistic monoclonal antibodies or antibody fragments against CD200 which either interfere with the CD200:CD200R interaction to block CD200R-mediated immune suppression or directly target CD200 expressing tumour cells by marking them for ADCC/CDC or by using a fusion molecule of the anti-CD200 mAb with a toxin (patent: Bowdish et al., 2007, 2009). Furthermore, a combination therapy with anti-CD200 mAb and anti-CD20 mAb (or a CD200 and CD20 bispecific antibody) is suggested for CLL treatment (Rother and Yan, 2011). One of the published CD200 blocking mAbs, ALXN6000, was taken further into a phase I/II study to determine safety and best dose for the treatment of relapsing or refractory B-CLL or multiple myeloma. The study was completed in November 2010 but no official outcome was presented so far.

2.12. A2aR

Soluble immune suppressive mediators can also be found in the tumour microenvironment and extracellular adenosine has been shown to be a critical and non-redundant negative regulator of a variety of immune cells (Hasko et al., 2008; Idzko et al., 2014; Ohta et al., 2006; Ohta and Sitkovsky, 2001). During the process of inflammation, the destruction of host tissue by the immune cells combined with damaged microcirculation and hypoxia leads to an increase in extracellular ATP levels (Sitkovsky, 2003; Sitkovsky et al., 2004). A2A adenosine (purinergic) receptors (A2aR) were identified as

sensors of excessive inflammatory tissue damage (Ohta and Sitkovsky, 2001) since normally nonlethal inflammation in wt mice led to excessive inflammation and death in *A2aR*-null mice (Ohta et al., 2006).

The tumour microenvironment also presents high levels of adenosine, as a result of hypoxia and ectopic expression of CD39 and CD73 ectonucleotidases responsibles for the generation of adenosine from ATP by Tregs (Deaglio et al., 2007; Stagg and Smyth, 2010; Young et al., 2014). Adenosine can then interact with four G-protein-coupled receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) with variable affinity, thus allowing differential responses to adenosine levels (Olah and Stiles, 1995). Interestingly, in both human and mice, A2aR proteins and their mRNA levels are preferentially upregulated in $CD4^+$ rather than upon activation (Lappas et al., 2005; Li et al., 2012; Koshiba et al., 1999). A2A adenosine receptors are potent inhibitors of T-cell receptor (TCR)-triggered proliferation as well as upregulation of interleukin-2 receptor (Huang et al., 1997; Sitkovsky et al., 2004). Indeed, A2aR signalling to $CD4^+$ and $CD8^+$ T cells has been shown to limit their effector function (Erdmann et al., 2005; Fishman et al., 2009; Lappas et al., 2005). A2aR engagement can prevent the development of IL-17 producing cells and promote the development of $Foxp3^+$ and LAG-3^+ regulatory T-cells *in vivo* (Zarek et al., 2008; Deaglio et al., 2007). Thus, Ohta and colleagues hypothesised that A2aR could protect cancerous cells by inhibiting the activity of tumour-reactive lymphocytes. Experiments using *A2aR*-null mice demonstrated an increased rejection of transplantable melanoma and lymphoma (Ohta et al., 2006) as well as a delay in the EL4 lymphoma cells growth associated with increased efficacy of tumour vaccines (Waickman et al., 2012). Activation of the A2aR results in inhibition of the immune response to tumours through several mechanisms, including suppression of T regulatory cell function, inhibition of natural killer cell cytotoxicity and tumour-specific $CD4^+$/$CD8^+$ activity (Fishman et al., 2009). Adenosine inhibits production of various cytokines and chemokines (IFN-γ, IL-2, TNF-α, GM-CSF, MIP-1α, MIP-1β, IL-13, and RANTES) in $CD8^+$ and $CD4^+$ Th1 cells, indicating that adenosine affects a common regulatory mechanism in their production (Raskovalova et al., 2007). The use of a specific agonist of A2aR indicates that the inhibitory effects of adenosine are mediated via increased levels of cyclic AMP (cAMP), leading to protein kinase A (PKA) activation (Raskovalova et al., 2007). The rise in intracellular cAMP does not only inhibit Th1 and Th17 cell generation but also promotes the generation of $Foxp3^+$ and LAG-3^+ regulatory T cells, hence producing a self-amplifying regulatory loop within the tumour (Zarek et al., 2008).

To conclude, A2aR-adenosine binding enhances polarisation of T-cell subsets to immunosuppressive phenotypes, leading to increase tumour growth. Therefore, pharmacological inhibition of A2aR activation by specific antagonists or antibodies that block adenosine binding is likely to synergise with other immunotherapies. Although this target has not yet been evaluated in cancer patients, the effect of agonistic antibodies to A2aR is currently been evaluated in atherosclerotic cardiovascular disease, and autoimmune syndromes such as systemic lupus erythematosus and rheumatoid arthritis (NCT01180361).

3. Conclusions and future perspectives

Essential for immune homoeostasis, inhibitory checkpoints predominate in the presence of active malignancy, and prevent effective anti-tumour immune responses. Research over the past ten years has highlighted that manipulation of these immune checkpoints with therapeutic antibodies can control or even eliminate tumours, leading to novel approaches to tumour immunotherapy. Antibodies that block inhibitory pathways, such as those directed against CTLA-4 and PD-1 have been developed for clinical use and have resulted in promising clinical outcomes, exemplified by their FDA approval. Future studies should delineate the mechanisms underpinning immunologic checkpoint function within diverse tumour microenvironments, and provide the rationale for effective personalised therapeutic combinations with other approaches such as chemotherapy, radiotherapy, and targeted therapies. Gene editing strategies now also allow consideration of targeting these inhibitory pathways in a variety of cell therapies including TILs, CIK cell (cytokine induced killer), CAR – (chimeric antigen receptors) or TCR-engineered T cells. Such cell-intrinsic disruption of immune checkpoints in tumour-specific T-cells may display a better safety profile than the systemic administration of blocking antibodies.

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We note that not all relevant results have been discussed and cited, owing to space limitations.

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