

Perspectives for immunotherapy in endocrine cancer

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Abstract

The fight against cancer has seen major breakthroughs in recent years. More than a decade ago, tyrosine kinase inhibitors targeting constitutively activated signaling cascades within the tumor inaugurated a new era of oncological therapy. Recently, immunotherapy with immune checkpoint inhibitors has started to revolutionize the treatment of several malignancies, most notably malignant melanoma, leading to the renaissance and the long-awaited breakthrough of immuno-oncology. This review provides an overview of the basis of immunotherapy from its initial concepts of anti-tumor immunity and cell-based therapy to the development of immune checkpoint inhibitors and discusses published studies and the perspectives of immuno-oncology for the treatment of endocrine malignancies.

Key Words

- ▶ immuno-oncology
- ▶ immune checkpoint blockade
- ▶ anti-CTLA-4
- ▶ anti-PD-1
- ▶ tumor microenvironment
- ▶ endocrine and neuroendocrine cancer

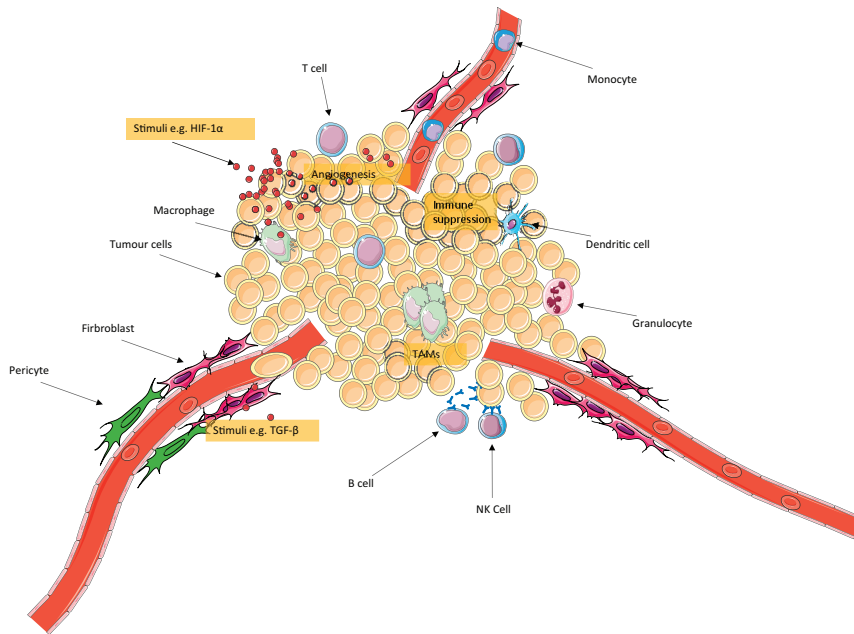
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Introduction

For many years, the target of anticancer therapy was the tumor cell itself. This was supported by the perception that cancer is defined as autonomously proliferating cells and is the rationale for traditional chemotherapy. Subsequently, the identification of mutated oncogenes leading to constitutively activated signaling pathways within a tumor not only improved understanding of carcinogenesis but led to the development of targeted therapies, in particular tyrosine kinase inhibitors (TKIs). TKIs have indeed changed treatment of cancers in which chemotherapy was ineffective, including endocrine malignancies such as radioiodine refractory thyroid cancer (Brose *et al.* 2014, Schlumberger *et al.* 2015), medullary thyroid cancer (MTC) (Wells *et al.* 2012, Elisei *et al.* 2013) and neuroendocrine tumors of the pancreas (Kulke *et al.* 2008, Raymond *et al.* 2011). TKIs counteract tyrosine

kinase signaling, for example through RET, EGFR, MET, VEGFR, BRAF and PI3K/AKT and usually affect multiple pathways. The extent to which the benefits of TKIs are due to their effect on tumor signaling *per se*, or are due to anti-VEGF effects that shut down tumor vasculature, is still a matter of debate. Either way, TKIs confer disease stabilization or at best partial response but do not allow for cure of solid cancers (Levitzi 2013).

In addition, the concept of an exclusive oncogene addiction of cancer has been challenged by evidence that tumour cells are in perpetual interaction with their microenvironment, also termed the ‘tumor stroma’. The tumor stroma describes the non-cancerous cells present in or adjacent to the tumor and in principle consists of distinct mesenchymal cells, tumor vessels, extracellular matrix and immune cells (Fig. 1)

**Figure 1**

Scheme of tumor microenvironment containing tumor cells, tumor vessels, pericyte support fibroblast and cells of innate and adaptive immune system. HIF-1 α , hypoxia-inducible factors; TAMs, tumor-associated macrophages; TGF- β , transforming growth factor- β .

(Klement 2016). In this microenvironment, the tumor gains 10 characteristic features that were defined by Hanahan and Weinberg as 'The Hallmarks of Cancer' (Hanahan & Weinberg 2000, 2011): (i) resisting cell death, (ii) sustaining proliferative signaling, (iii) evading growth suppressors, (iv) enabling replicative immortality, (v) inducing angiogenesis, (vi) activating invasion and metastasis, (vii) deregulation of cellular energetics, (viii) genome instability and mutation, (ix) tumor-promoting inflammation and (x) avoiding immune destruction.

In recent years, researchers have focused on dissecting components of this interplay, in particular the role of the immune system. These studies have led to the development of immunotherapies that may induce, enhance or suppress an immune response against cancer cells. Decades ago, the first concepts were developed to reach these goals, for example with IL-2 (Pizza *et al.* 1988), IL-7 (Moller *et al.* 2000) or interferons (Vadhan-Raj *et al.* 1986); with activating immunotherapy, for example by T-cell adoptive transfer (Curti *et al.* 1993) or dendritic cell (DC) vaccination (Gjertsen *et al.* 1996) or alternatively with suppressive immunotherapy, for example by glucocorticoids (Vietti *et al.* 1965). In this review, we will briefly introduce these concepts and the role of immune checkpoint inhibitors, which are currently recognized as a major breakthrough in immuno-oncology, before we review the published literature on immunoenvironment and immunotherapy in endocrine cancers (Table 1).

Initial concepts of immunotherapy using antibodies directed against cancer cells

In immunomodulation, an antibody is directed against the tumor cell itself, conferring apoptosis or antibody-dependent cellular cytotoxicity (ADCC). This kind of monoclonal antibody is designed to bind to a specific surface molecule, for example a tumor-associated antigen to ensure tumor-specific effects. The first antibody used to treat cancer, rituximab (Mabthera), was approved by the US Food and Drug Administration (FDA) in 1997. Rituximab is a specific CD20 antibody, targeting CD20+ B cells in leukemia and malignant lymphoma. The FDA approval of rituximab was followed by the approval of denileukin diftitox (Ontak) for targeting cutaneous T cells 2 years later. Denileukin diftitox is a fusion protein in which the native receptor-binding domain of diphtheria toxin is replaced by the full-length interleukin-2 (IL-2). This modification allows binding of the drug to IL-2-R expressing T cells (especially the α -chain of the receptor (CD25)), introduction of diphtheria toxin into the cells and consequently induction of apoptosis. Preclinical and clinical studies indicate that stage IV melanoma patients might benefit from depletion of regulatory T cells (Treg) (Mahnke *et al.* 2007, Telang *et al.* 2011). However, effects of denileukin diftitox did not correlate with CD25 expression and Treg depletion was not confirmed unambiguously (Attia *et al.* 2005). The major disadvantage of this and other early drugs designed for similar targets was their lack of specificity

Table 1 Literature on tumour microenvironment and immunotherapy in endocrine/neuroendocrine cancers.

Tumour	Model/tissue	Technique	Result	Literature
PTC, fvPTC, tcPTC, FTC (minimal and widely invasive), FA, nodular goiter, normal tissue	FFPE	IHC	TAM increases with dedifferentiation	Cunha et al. (2012)
PTC, fvPTC, tcPTC, FTC (minimal and widely invasive), FA, nodular goiter, normal tissue	FFPE	IHC, mRNA level	PD-L1 expression correlates with higher CD4+, CD8+, CD20+ and FoxP3+ lymphocyte content and TAM infiltrate	Cunha et al. (2013)
PTC, autoimmune thyroiditis	Frozen tissue section	IF, FACS	CD3+, CD4- and CD8- were significantly more abundant in thyroid cancer compared to thyroid autoimmunity	Imam et al. (2014)
DTC, PDTC, ATC	FFPE	IHC, microarray	TAM density increased in advanced thyroid cancers and correlated with invasion and decreased patient survival	Ryder et al. (2008)
FTC, FA	FFPE	IHC	Higher density of TAMs increases chemokine CCL15	Huang et al. (2015)
PDTC, ATC	FFPE	NGS	Macrophage gene expression pattern was sufficient to discriminate ATC from PDTC	Landa et al. (2016)
PTC	FFPE	IHC	BRAF ^{V600E} tumours express high levels of PD-L1 (53% vs 12.5%) compared to BRAF WT tumours, no significant difference in TAM pattern was observed	Angell et al. (2014)
PTC, ATC, normal tissue	Human tissue microarray sections	Microarray	ATCs had the highest density of CD163-positive macrophages (22.9 ± 17.1%), PTC the lowest (1.8 ± 1.3%)	Jung et al. (2015)
MTC	Patients	Peptide-pulsed DC vaccination	Response rate 3/7 patients	Schott et al. (2001)
MTC	Patients	Peptide-pulsed DC vaccination	Response rate 3/10 patients	Bachleitner-Hofmann et al. (2009)
MTC	Patients	<i>Ex vivo</i> generated granulocyte-macrophage colony-stimulating factor and interferon-alpha dendritic cells	Response rate 2/5 patients	Papewalis et al. (2008)
GEP-NETs (distinct locations)	FFPE	IHC	PD-L1 is a negative prognostic factor in GEP-NETs	Kim et al. (2016)
PTC (inducible)	BRAF ^{V600E} mouse model	Intramuscular injection of mrIL-12 plasmid and intraperitoneal injection of mrIL-12, IHC, NK cell assay and phenotyping of effector cells	Significantly lower tumor burden mediated by CD8+ and natural killer cells	Parhar et al. (2016)
ATC	Genetically engineered murine ATC: TBP 3743 BRAF ^{V600E} /WT P53 ^{-/-} cells were implanted into immunocompetent mouse thyroid	tumour volume	Combined treatment of BRAF-inhibitor and PD-L1 antibody (baseline: 782.3 ± 174.6 mm ³ vs 439.3 ± 188.4 mm ³ (BRAF inhibitor) and 716.7 ± 62.1 mm ³ (PD-L1) to 147.3 ± 60.8 mm ³ with BRAF inhibitor/PD-L1 combination) was more efficient than single treatments	Brauner et al. (2016)
Pheochromocytoma	Mouse model	Chromogranin A-based dendritic cell vaccination	Chromogranin A vaccinated mice show a large amount of infiltrating CD8+ cells and reduction of liver metastases	Papewalis et al. (2011)

ATC, anaplastic thyroid cancer; FA, follicular adenoma; FFPE, formalin fixed paraffin embedded tissue; FTC, follicular thyroid cancer; fvPTC, follicular variant PTC; GEP-NET, gastrointestinal pancreatic neuroendocrine tumours; IF, immunofluorescence; IHC, immunohistochemistry; MTC, medullary thyroid cancer; PDTC, poorly differentiated thyroid cancer; PTC, papillary thyroid cancer; tcPTC, tall cell PTC.

resulting in an increasing number of severe adverse events (AEs), including a black box warning in some cases. Once TKI emerged, the number of trials exploring this approach for solid malignancies decreased. Nevertheless, immunomodulation continues to play a role in the treatment of hematological malignancies: breast (anti-HER2), colorectal and head and neck cancer (cetuximab).

Cell-based therapies

Effective anti-tumor immunity is based on the existence of a repertoire of the so-called 'autoreactive' T and B cells. When these cells are properly activated, they can recognize malignant cells, which express tumor-associated antigens (Coulie et al. 2014).

Adoptive T-cell transfer of tumor-infiltrating lymphocytes (TILs) is a cell-based immune therapy and involves *ex vivo* expansion of specific effector cells. Pilot studies for *ex vivo* expansion of TIL were already done in the 1980s in melanoma, renal cell, breast and colon carcinoma patients, and indicated that melanoma is a more immune-sensitive cancer than others (Topalian et al. 1988). Efficiency of TIL therapy can be enhanced by applying IL-2 to the patients. Preparative lymphodepleting chemotherapy of melanoma patients followed by high-dose IL-2 in combination with TIL conferred a 50% response rate in clinical trials and it seemed that a subset of patients has durable benefit from this treatment (Dudley et al. 2005, Besser et al. 2010, Wu et al. 2012). TIL therapy, *per se*, causes minor side effects, but in combination with IL-2 severe AEs were observed such as hypotension, diarrhea, chills, vomiting, nausea, confusion and vascular leak syndrome (Dreno et al. 2002, Kradin et al. 1989, Rosenberg et al. 1989). TIL therapy is a laborious, personalized approach, which cannot be applied off-the-shelf and hence it is currently only explored in clinical trials.

DC-based vaccination follows a different proactive route of cancer treatment. In DC vaccination, it is possible to use a multi-peptide vaccination cocktail against several antigens *in vivo*. If DC takes up and processes those peptides, it is possible to prime a larger number of specific lymphocytes with a diverse T-cell receptor (TCR) repertoire (Jeanbart & Swartz 2015). Thus, the principle of DC vaccination is to 'teach' the immune system to kill tumor cells that express specific peptides or cocktails of peptides on their surface. Researchers focusing on this approach have to meet several challenges: One problem is to find immunogenic peptides expressed only on the

surface of the tumor cells to avoid adverse effects in the periphery. Another challenge is the effective and fast priming of effector cells *in vivo*. So far, no randomized clinical trial has been able to show efficacy of a DC-based vaccine (Yi & Appel 2013), however, combinational usage of DC could be a promising future perspective. Recently, a first randomized phase II trial was launched to test DC vaccination in combination with TKI (Dasatinib) treatment in metastatic melanoma patients (NCT01876212). Results of this trial are awaited by July 2018.

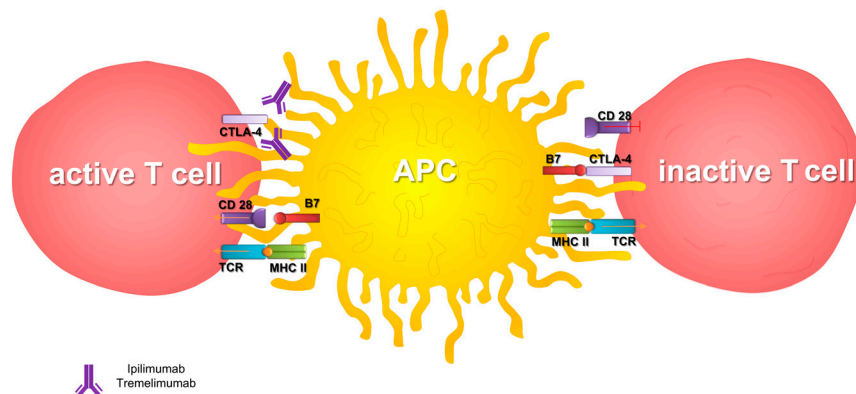
Immune checkpoint inhibitors: the milestone in immuno-oncology

Immune evasion mechanisms are crucial for tumor cell survival (Hanahan & Weinberg 2000, 2011). The pivotal role of CTLA-4 (Leach et al. 1996) and subsequently of programmed death receptor-1 (PD-1)/PD-L1 (Dong et al. 2002) in that respect has led to the development of antibodies against these molecules. These antibodies, the so-called 'immune checkpoint inhibitors', have inaugurated a new era of cancer treatment (Melero et al. 2015, Topalian et al. 2015).

CTLA-4 is expressed on the surface of helper, cytotoxic and regulatory T cells, and is a member of the immunoglobulin superfamily. CTLA-4 is, similar to CD28, a T-cell co-stimulatory protein. Both are able to bind to B7 family members, which are expressed on antigen-presenting cells (APCs). While CD28 ligation is a co-stimulatory signal for TCR activation, CTLA-4 binding results in T-cell inhibition (Fig. 2). *In vivo*, TCR binding leads to upregulation of CTLA-4, which has a higher affinity to B7 molecules than CD28. By adding a CTLA-4 antibody as a checkpoint inhibitor the negative feedback is decreased and the T-cell cytotoxic response augmented (Fig. 2) (Walunas et al. 1994, Krummel & Allison 1995, Waterhouse et al. 1995, Walunas et al. 1996).

In vivo experiments in rodents demonstrated that anti-CTLA-4 therapy leads to reduction in size of several types of cancer, such as colon (Leach et al. 1996, Son et al. 2014), renal (Lan et al. 2013) and prostate cancer (Kwon et al. 1997, Li et al. 2014) and lymphoma (Shrikant et al. 1999, Sotomayor et al. 1999). Treatment with the anti-CTLA-4 antibody ipilimumab (Yervoy, Bristol-Myers Squibb, Princeton, NJ, USA) showed a significant benefit for overall survival in patients with advanced melanoma (Table 1) (Hodi et al. 2010, Robert et al. 2011). In a pooled analysis of more than 1800 patients with a follow-up period of up to 10 years, it was shown that ipilimumab leads to long-term survival in about 20% of

Lymph Node

**Figure 2**

T-cell activation in a lymph node after antigen presentation by APC via TCR/MHCII complex and co-stimulatory signal B7. Right: CTLA-4 binding to B7 together with TCR complex results in T-cell inactivation. Left: Anti-CTLA-4 antibodies block the interaction of CTLA-4 and B7 and thereby confer T-cell activation. MHC, major histocompatibility complex; TCR, T-cell receptor.

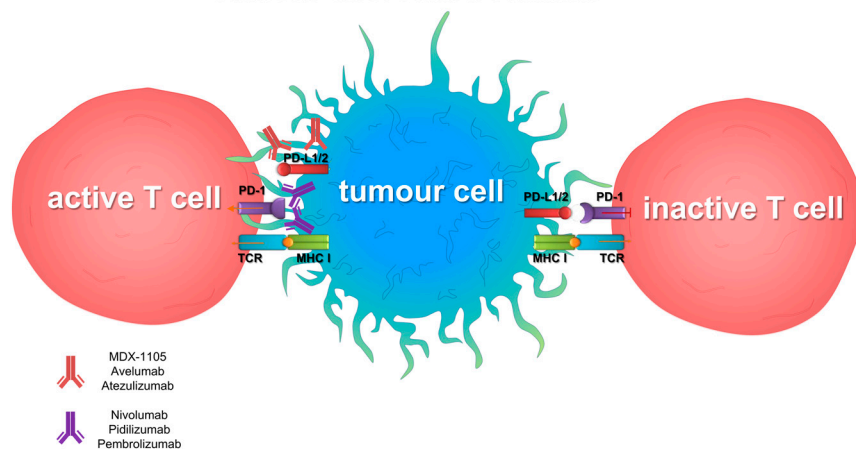
melanoma patients (Schadendorf *et al.* 2015). Ipilimumab was the first checkpoint inhibitor to be approved by the FDA in 2011 for unresectable stage III or metastatic stage IV melanoma and subsequently as adjuvant therapy for high-risk stage III melanoma in 2015 (Eggermont *et al.* 2015).

AEs related to anti-CTLA-4 treatment are frequent and include immune-related endocrinopathies, notably hypophysitis (Yang *et al.* 2007), adrenalitis (Yang *et al.* 2007) and thyroiditis (Blansfield *et al.* 2005, Dillard *et al.* 2010, Min *et al.* 2011, Corsello *et al.* 2013, Ryder *et al.* 2014), in addition to other immune-related AE such as colitis (Royal *et al.* 2010), hepatitis and uveitis (Hodi *et al.* 2014).

PD-1 is a cell surface molecule closely related to CTLA-4 and is part of the CD28 family. PD-1 inhibits immune response by preventing T-cell activation (Ishida *et al.* 1992). B cells, T cells, monocytes, natural killer cells and TILs express PD-1 on their surface. PD-1 interacts with two known ligands: PD-L1 and PD-L2 (Latchman *et al.* 2001). PD-L1 expression was initially reported on

melanoma, ovarian, colon and lung cancer cells (Dong *et al.* 2002) but can also be found in other human cancers such as breast (Baptista *et al.* 2016), head and neck (Malm *et al.* 2015) and pancreatic cancer (Bigelow *et al.* 2013). Interaction of PD-1 with PD-L1 has been conceived to act as an immune evasion mechanism for tumor cells (Dong *et al.* 2002, Iwai *et al.* 2002, Wu *et al.* 2006). Interestingly, antitumor immunity itself seems to drive PD-L1 expression on cancer cells (Spranger *et al.* 2013). Antibodies interrupting PD-1/PD-L1 interaction (Fig. 3) such as nivolumab (anti-PD-1, Bristol-Myers Squibb), pembrolizumab (anti-PD-1, MSD Merck, Whitehouse Station, NJ, USA) and atezulizumab (anti-PD-L1, Roche/Genentech, Basel, Switzerland) have recently been approved for the treatment of advanced melanoma, non-small cell lung cancer, advanced bladder cancer, Hodgkin lymphoma and metastatic renal cell carcinoma (Table 2). PD-1/PD-L1 antibodies are surprisingly well tolerated with mostly CTCAE grade I or II AE-like rash, itching and fatigue making their application particularly attractive

Tumour Microenvironment

**Figure 3**

Interaction of T cells with tumor cells within the tumor microenvironment. Right: Tumor cells expressing PD-L1/2 are able to inactivate T cells via PD-1 pathway. Left: Tumor cells lose this ability when PD-1 and/or PD-L1/2 are blocked by antibodies and an appropriate T-cell response occurs resulting in killing of tumor cells. MHC, major histocompatibility complex; TCR, T-cell receptor.

Table 2 Selected clinical trials on immune checkpoint inhibitors in human malignancies.

Trial design	Study population	Treatment arms	N	ORR (%)	Endpoints	Literature	FDA approval
CTLA-4							
Three-armed, randomized, double-blind, phase III	Unresectable stage III or IV melanoma after previous chemotherapy or interleukin-2	Ipilimumab + gp100 vaccine vs ipilimumab vs gp100 vaccine, randomized 3:1:1	676	5.7 vs 10.9 vs 1.5	Median OS: 10 vs 10.1 vs 6.4 months Grade 3+4 AEs: 10–15% with ipilimumab vs 3% with gp100 only	NCT0094653 (Hodi et al. 2010)	Yes
Two-armed, randomized, double-blind, phase III	Unresectable stage III or IV melanoma no prior systemic treatment	Dacarbazine + ipilimumab vs dacarbazine + placebo, randomized 1:1	502	15.2 vs 10.3	Median OS: 11.2 vs 9.1 months 1-year SR: 47.3% vs 36.3% 2-year SR: 28.5% vs 17.9% 3-year SR: 20.8% vs 12.2%	NCT00324155 (Robert et al. 2011)	No
Two-armed, randomized, open-label, phase III	Unresectable stage III or IV melanoma no prior systemic treatment	Tremelimumab vs ICC (temozolomide or dacarbazine), randomized 1:1	655	10.7 vs 9.8	Median OS: 12.6 vs 10.7 months ORR: 10.7% vs 9.8% RD: 35.8 vs 13.7 months	NCT00257205 (Ribas et al. 2013)	No
CTLA-4 plus PD-1 blockade							
Phase III	Unresectable stage III or IV melanoma, no prior systemic treatment	Nivolumab plus ipilimumab or ipilimumab vs ipilimumab, randomized 1:1:1	945	57.6 vs 43.7 vs 19	Median PFS: 11.5 vs 6.9 vs 2.9 months Grade 3+4 AEs: 55% vs 16.3% vs 27.3% OS not yet available	NCT01844505 (Larkin et al. 2015)	Yes (2015)
PD-1/IPD-L1							
Three-armed, randomized, phase III	Unresectable stage III or IV melanoma, maximum of one prior systemic treatment	Pembrolizumab (10 mg/kg) every 2 weeks or pembrolizumab (10 mg/kg) every 3 weeks vs ipilimumab (3 mg/kg, 4 cycles), randomized 1:1:1	834	33.7 vs 32.9 vs 11.9	Hazard ratio for disease progression 0.58 for both pembrolizumab regimens vs ipilimumab, hazard ratio for death for pembrolizumab every 2 weeks, 0.63 vs ipilimumab and 0.69 for pembrolizumab every 2 weeks vs ipilimumab 1-year SR: 72.9% vs 42.1%	NCT01866319 (Robert et al. 2015b)	Yes
Two-armed, randomized, double-blind, phase III	Unresectable stage III or IV melanoma, no prior systemic treatment, BRAF wild type	Nivolumab (3 mg/kg) every 2 weeks vs DTIC (1000 mg/m ²), randomized 1:1	418	40.0 vs 13.9		NCT01721772 (Robert et al. 2015a)	Yes
Three-armed, randomized, open-label, phase II/III	Previously treated, advanced NSCLC, PD-L1 positive	Pembrolizumab (2 mg/kg) or pembrolizumab (10 mg/kg) vs docetaxel (75 mg/m ²), randomized 1:1:1	1034	18 vs 18 vs 9	Median OS: 10.4 vs 12.7 vs 8.5 months Median PFS: 3.9 vs 4.0 vs 4.0 months	NCT01905657 (Herbst et al. 2016)	Yes (for tumors with at least 50% cells positive for PD-L1 in companion diagnostics test)

Three-armed, randomized, open-label, phase III	NSCLC, progressed after/during platin-based chemotherapy	Nivolumab (3 mg/kg) every 2 weeks vs docetaxel (75 mg/m ²), randomized 1:1	582	19 vs 12	Median OS: 12.2 vs 9.4 months Median PFS: 4.2 vs 2.3 months 1-year SR: 51% vs 39%	NCT01673867 (Borghaei et al. 2015)	Yes
Two-armed, randomized, open-label, phase III	Previously treated, advanced Renal-Cell Carcinoma	Nivolumab (3 mg/kg) every 2 weeks vs 10 mg everolimus daily, randomized 1:1	821	25 vs 5	Median OS: 25.0 vs 19.6 months, Median PFS 4.6 vs 4.4	NCT01668784 (Motzer et al. 2015)	Yes
Single-arm, phase II	Locally advanced or metastatic bladder (urothel) cancer failing platinum-containing chemotherapy	Atezolizumab (1200 mg) every 3 weeks	310	15	Median OS 7.9 months 1-year SR: 36%	NCT02108652 IM vigor 2:10 trial, not published	Yes (contingent)
Single arm, phase I	Relapsed or refractory Hodgkin's lymphoma	Nivolumab (3 mg/kg) every 2(3) weeks	23	87	Related AE: Grade 3 or higher: 22% Median OS: not reached 24-weeks PFS rate: 86% 18.8	NCT01592370 (Ansell et al. 2015)	Yes
Single-center, single arm, phase II	Patients with rituximab-sensitive follicular lymphoma	Pidilizumab (3 mg/kg) with rituximab (375 mg/m ²)	32	66		NCT00904722 (Westin et al. 2014)	No

DR, duration of response; ICC, investigator choice chemotherapy; N, number of patients analysed; ORR, overall response rate; OS, overall survival; PFS, progression-free survival.

(Hofmann et al. 2016, Zimmer et al. 2016). In about 10–15% of patients receiving these antibodies, higher grade AEs such as colitis, hepatitis and pneumonitis do occur.

Little is known so far on PD-L2 function, except that it is expressed on the surface of DCs, macrophages, mast cells and B lymphocytes (Topalian et al. 2012). PD-L2 is upregulated in follicular B-cell lymphoma, Hodgkin lymphoma and primary mediastinal B-cell lymphoma (Rosenwald et al. 2003). Recent research could also identify an overexpression of PD-L2 in more than 50% of breast cancer samples (Baptista et al. 2016) and esophageal squamous cell carcinoma but no correlation was found with disease-free survival and/or overall survival (Leng et al. 2016). So far, no therapeutics directly target PD-L2.

Recently, results of a randomized, double-blind, phase III head-to-head study were published comparing nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) alone or in combination with previously untreated stage IV melanoma patients. Combination therapy resulted in significantly longer progression-free survival (PFS) (11.5 months) compared with nivolumab (6.9 months) or ipilimumab (2.9 months) monotherapy (Larkin et al. 2015). Unfortunately, severe grade 3 or 4 AE occurred in the combination treatment arm in 55% of patients, illustrating the narrow gap between effective anti-tumor response and unwanted side effects. However, several trials are currently exploring the use of combined immune checkpoint inhibitors (Antonia et al. 2016a,b) or the combination of immune checkpoint inhibitors together with other anticancer drugs such as chemotherapy or TKI (listed in Table 2) and the results of these will provide important information on how to best achieve antitumor treatment with tolerable side effects.

As immune checkpoint inhibitors do not appear to work for all cancers, with adenocarcinoma of the pancreas being a prominent example of lack of response (Royal et al. 2010), one crucial and also economically relevant issue is to define biomarkers that will predict response to checkpoint inhibitor therapy. Currently, response to immunotherapy seems to be dependent on various determinants, which indicates that a single predictive biomarker for successful immunotherapy of cancer is unlikely to be defined (Blank et al. 2016). Several studies have shown a correlation of tumor PD-L1 expression and response to anti-PD-1/PD-L1 treatment (Herbst et al. 2014, Borghaei et al. 2015, Garon et al. 2015, Weber et al. 2015), whilst others did not (Larkin et al. 2015, McDaniel 2015, Robert et al. 2015b). Furthermore, one methodological problem using PD-L1 as a single biomarker might be that different antibodies are

used for immunohistochemistry (IHC) including but not limited to clones 28-8, 22C3, SP142, SP263, 5H1, MIH1 and 405.9A11. Furthermore, membrane and cytosolic staining for PD-L1 have to be distinguished (Joseph Grosso 2013, Angell *et al.* 2014, Robert *et al.* 2015a,b). In addition, harmonization of IHC protocols and definition of reliable cut-offs for quantification of PD-L1 staining is still missing with most tests focusing on PD-L1 expression on tumor cells (Borghaei *et al.* 2015) while others also include myeloid cells (Herbst *et al.* 2014). An additional challenge with analysis of PD-L1 expression is that the pattern may be dynamic due to adoption and interplay of tumor cells and their microenvironment, which may change with tumor evolution and progression (Fusi *et al.* 2015).

In the melanoma setting, apart from PD-L1 expression in the tumor microenvironment, density of CD8+ T-cell infiltrates has been found to be associated with clinical benefit from PD-1 blockade (Tumeh *et al.* 2014). Moreover, it was recently reported that high relative eosinophil count (>1.5%), high relative lymphocyte count (>17.5%) and low serum lactate dehydrogenase (<2.5× elevation) were associated with response to pembrolizumab in melanoma patients (Weide *et al.* 2016) and a similar association has also been described for ipilimumab (Martens *et al.* 2016). In melanoma patients, these biomarkers may help to guide treatment decision, in particular as in BRAF^{V600E} positive tumors, first-line therapy could involve either MAPK inhibitors (Larkin *et al.* 2014, Long *et al.* 2014) or a checkpoint inhibitor (Larkin *et al.* 2015, Robert *et al.* 2015b). An excellent overview of potential biomarkers predicting response to checkpoint inhibitor therapy has recently been published by Manson *et al.* (2016).

In addition, genetic alterations within the tumors also need to be considered. Overall, mutational load has been found to be associated with benefit from immune checkpoint blockade in melanoma and squamous cell lung cancer (Rizvi *et al.* 2015, Van Allen *et al.* 2015), especially when mutations were clonal (McGranahan *et al.* 2016). This finding can be explained by somatic mutations giving rise to the so-called neoantigens that act as tumor antigens. Hence, highly mutated tumors do look more foreign to the autologous immune system and are more prone to be recognized by T cells (Schumacher & Schreiber 2015). This reasoning is also sustained by Champiat *et al.* in their meta-analysis of checkpoint inhibitor trials and treatment outcomes, which were correlated with the degree of genomic instability in the tumor. Based on this analysis they proposed that 'high levels of mutational heterogeneity and thus genomic

instability in the tumor could be the key for success of immune checkpoint therapies' (Champiat *et al.* 2014).

Imaging of oncological patients undergoing immunotherapy: immune-related response criteria

While the response evaluation criteria in solid tumors (RECIST) are an internationally accepted tool to determine tumor response (Eisenhauer *et al.* 2009), these criteria were developed when oncologic treatments directly targeted proliferating tumor cells. With immunotherapy, however, objective response often manifests after initial tumor mass increase, due to immune cell infiltration, often called pseudo-progression, or the appearance of new lesions. Therefore, application of RECIST criteria might underestimate the therapeutic benefit of checkpoint inhibitors (Wolchok *et al.* 2009). In 2009 immune-related response criteria were first proposed for ipilimumab therapy in malignant melanoma patients (Wolchok *et al.* 2009) and were subsequently evaluated also for pembrolizumab treatment (Hodi *et al.* 2016). This issue has also been discussed in neurooncology (Okada *et al.* 2015) and for non-small cell lung cancer (Socinski 2015). A comprehensive overview of the application and relevance of immune-related response criteria is given by Hoos *et al.* (2015).

Is there a role for immunotherapy in endocrine malignancy?

Endocrine tumors originate from cells that secrete hormones into the bloodstream and by this can cause serious illness as illustrated in patients with Cushing's disease, acromegaly or Heding syndrome. With few exceptions, endocrine malignancies are slowly proliferating cancers where TKI treatment results in disease stabilization or at best partial response. Classical chemotherapies that induce DNA damage and stop cells' ability to grow and divide are, therefore, not very effective. Cancer cells with slow accumulation of mutations may have a higher chance in successfully evading the immune system (Corthay 2014).

Thyroid cancer

A successful immune response depends largely on the recruited immune cells within the tumor. In recent years, different groups have characterized the immune microenvironment in thyroid cancer.

Cunha *et al.* analyzed samples of 398 patients with thyroid tumors (follicular thyroid carcinoma (FTC),

papillary thyroid carcinoma (PTC), FA) and normal tissues by IHC (Cunha *et al.* 2012). Tumor-associated macrophages (TAMs) were found in malignant (82.11%) and less frequently in benign thyroid tumors (33.91%). This may suggest that macrophages might be important in the context of thyroid cancer. Macrophages are part of the innate immune system and are divided into different subpopulations. The classically activated M1 macrophages are usually induced by proinflammatory signals (e.g. LPS), whereas M2 macrophages are activated due to wound healing or tissue repair, mainly by IL-4 and IL-13. TAMs are a subpopulation of macrophages that share both features and, therefore, are M1- and M2-like. TAMs are reported to possess pro- and antitumoral activity. Typical markers for TAMs are the cell surface proteins CD68 and CD163. Whereas CD68 (glycoprotein) is a relatively nonspecific marker for monocyte/macrophage lineage in melanoma, lymphoma and schwannoma (Nguyen *et al.* 2005), CD163 (hemoglobin scavenger receptor) has been shown to be highly specific for monocyte/macrophage lineage in the tumor (Nguyen *et al.* 2005, Harris *et al.* 2012).

Ryder *et al.* showed that the density of TAMs in human samples correlates with thyroid dedifferentiation. Thus, macrophage density increased from 27% in differentiated thyroid cancers to 54% in poorly differentiated thyroid cancer (PDTC) and reached 95% in anaplastic thyroid cancer (ATC). Furthermore, it was shown that high density of TAMs correlates with capsular invasion, extra-thyroidal extension and decreased cancer-related survival compared with low-density TAM concentrations in PDTC (Ryder *et al.* 2008). Similar results were reported by Jung *et al.* investigating the density of CD163+ macrophages in ATC ($22.9 \pm 17.1\%$, $N=18$) and PTC ($1.8 \pm 1.3\%$, $n=35$) (Jung *et al.* 2015). In a recent landmark paper on the genomic signature of PDTC and ATC, Landa *et al.* showed that 68 out of 78 investigated M2 macrophage genes were overexpressed in PDTC and ATC samples. Importantly, the authors report that the presence of macrophage infiltration was sufficient to even discriminate ATCs from PDTCs (Landa *et al.* 2016). Still the question remains: which mechanisms lead to macrophage recruitment? In this respect, Huang *et al.* studied FTC and FA samples and observed a higher density of TAMs in FTC (density of CD68+ cells $9.5 \pm 5.4/\text{field}$) compared with follicular adenoma ($4.9 \pm 3.4/\text{field}$), which correlated with increase of chemokine mRNA CCL15 (51-fold) expression and CCL15 immunostaining (68% in FTCs compared with 23% in FAs) (Huang *et al.* 2015).

In a recent study Cunha *et al.* also investigated PD-L1 expression in thyroid cancer (253 PTC, 40 FTC) and 114 benign thyroid tissues (58 nodular goiters and 56 follicular adenomas) and found that PD-L1 is expressed in 78.4% of goiters, 84.3% of follicular adenomas, 82.5% of PTCs and 87.5% of FTCs. No correlation was observed between PD-L1 expression and tumor size or cancer aggressiveness. However, higher expression of PD-L1 correlated with significant increase of CD4+ ($P=0.04942$), CD8+ ($P=0.0003$), CD20+ ($P=0.01283$), FoxP3+ ($P=0.00626$) lymphocytes and TAMs ($P<0.0001$) in the respective tumor samples. Interestingly, decreased PD-L1 expression was found in thyroid cancer lymph node metastases (Cunha *et al.* 2013).

Angell *et al.* studied 33 PTC for correlation between *BRAF* mutation status and PD-L1 expression using IHC and found that presence or absence of the *BRAF*^{V600E} mutation was associated with PD-L1 overexpression (53% *BRAF*^{V600E} (positive) vs 12.5% *BRAF*^{V600E} (negative)) and lower CD8+ effector to Foxp3+ regulatory T-cell ratios (8.67 ± 2.23 vs 30.32 ± 8.84 , respectively ($P<0.05$)). In contrast, *BRAF*^{V600E} positivity had no impact on TAM (CD68+ or CD163+ positive macrophages) content in PTCs (Angell *et al.* 2014). This observation leads to the hitherto unresolved question whether mutational features and/or load (e.g. RET, RAS, ALK, etc.) in thyroid cancer cells may correlate with PD-L1 overexpression.

Further aspects of the immune microenvironment of the thyroid gland were described by Imam *et al.* Challenging the notion that autoimmune thyroiditis and PTC may be linked, they compared the tumor-associated lymphocyte pattern in PTC ($n=11$) with lymphocytes found in autoimmune thyroiditis ($n=7$). Based on investigation of frozen tissue sections and flow cytometry (Imam *et al.* 2014), they showed that CD3+, CD4- and CD8- lymphocytes were significantly more abundant in PTC (>20%) compared with autoimmune thyroiditis (<5%). Moreover, after PMA/Ionomycin stimulation, the population of CD3+, CD4- and CD8- lymphocytes derived from PTC patients remained unchanged, but increased in samples derived from thyroid autoimmunity patients. Furthermore, CD25 (α -chain of the IL-2 receptor and marker for activation) expression on CD3+ and CD4+ did not change after stimulation in PTC and thyroid autoimmunity patients, leading the authors to conclude that the expansion of total numbers of CD3+, CD4- and CD8- lymphocytes in thyroid autoimmunity was at the expense of inactivation of single positive T cells.

One reason for the increase of CD3+, CD4- and CD8- lymphocytes in PTC might be loss of CD4 and

CD8 expression. This could indicate that CD3+, CD4- and CD8- lymphocytes play a role in downregulation of proliferation and cytokine production of activated effector T cells within the tumor microenvironment and may thereby play a crucial role in immune tolerance.

Various thyroid cancer mouse models have been developed in recent years including the inducible BRAF^{FV600E} mouse for PTC (Knauf *et al.* 2005) or an orthotopic mouse model for FTC (Greco *et al.* 2016). In such mouse models, it is now possible to evaluate the efficacy of immune checkpoint inhibitors and combined therapies for treatment of aggressive thyroid cancer. This approach has recently been shown to result in dramatically improved tumor regression under combined BRAF inhibitor and PD-L1 antibody treatment (baseline: 782.3±174.6 mm³ vs 439.3±188.4 mm³ (BRAF inhibitor) and 716.7±62.1 mm³ (PD-L1) to 147.3±60.8 mm³ with BRAF inhibitor/PD-L1 combination) (Brauner *et al.* 2016). In addition, a very recent study from Parhar *et al.* demonstrated the influence of IL-12 on immune response and tumor growth in BRAF^{FV600E} mice. They compared intramuscular injection of recombinant mouse IL-12 plasmid and intraperitoneal injection of recombinant mouse IL-12. Both treatments resulted in significantly lower tumor burden and were mediated by CD8+ and natural killer cells. Suppression of IL-12 abolished these effects indicating that IL-12 might be of interest as adjuvant therapy in advanced BRAF^{FV600E} thyroid cancers (Parhar *et al.* 2016).

With the help of these preclinical models, it will be possible to further phenotype and characterize the development and recruitment of immune cells during thyroid carcinogenesis and cancer progression as a prerequisite to define successful immunotherapy. In this respect, TAMs that show high expression of immunosuppressive cytokines (e.g. IL-10, TGFB1) may be attractive, but PD-1/PD-1L *per se* could be a suitable target as well. In addition, following the hypothesis of Champiat and coworkers and in view of the findings recently published by Landa and coworkers showing increased mutational load in PDTC and ATC, it is conceivable that immunotherapy may be useful in these most detrimental TC (Champiat *et al.* 2014, Landa *et al.* 2016).

Clinical trials

Immunotherapy for treatment of thyroid cancer was tested in pilot studies in few patients with MTC and advanced differentiated thyroid cancer. Schott *et al.*

showed that calcitonin and/or carcinoembryonic antigen peptide pulsed DC resulted in a cellular antigen-specific immune response in some MTC patients (Schott *et al.* 2001). Similar results were obtained in a study by Bachleitner-Hofmann *et al.* (2009). Improvement of this technique led to *ex vivo* generated granulocyte-macrophage colony-stimulating factor and interferon-alpha DCs (IFN-DCs) that were injected into 5 patients. Two patients had an excellent response with a large increase of antigen-specific IFN-gamma-secreting CD4+ cells and CD8+ cells with increase of granzyme B in the peripheral blood. Interestingly, a decrease of CD4+, CD25+ and FoxP3+ regulatory T cells was observed (Papewalis *et al.* 2008). Wuttke *et al.* used a mouse model (Ret/Cal) with constitutive RET activation, where it was possible to detect a significant increase of peptide-specific CD8+ T cells after 6 months of immunization. Furthermore, it was possible to detect intratumoral infiltration of those CD8+ T cells and tumor outgrowth was reduced by approximately 57% (Wuttke *et al.* 2008).

Lysate pulsed monocyte-derived mature DCs vaccination for 8 weeks in combination with low dose IL-2 was also used in phase I clinical study (*n*=6) including PTC and FTC patients. No AEs were observed, in two patients stable disease was achieved compared with four patients suffering from progressive disease (Kuwabara *et al.* 2007). Unfortunately, in the recent years no further clinical trials were published using these approaches.

Gastrointestinal neuroendocrine tumors and pheochromocytoma

Gastrointestinal neuroendocrine tumors (GEP-NETs) are heterogeneous tumors originating from cells of the endocrine (hormonal) and nervous systems. The prevalence is estimated to reach 35 cases/100,000 per year (Oberge *et al.* 2012, Alonso-Gordoa *et al.* 2015). GEP-NETs share morphological characteristics and may lead to hormone excess. Available medical therapies include somatostatin analogues, interferon-alpha, TKIs, mTOR inhibitor and chemotherapy (Kotteas *et al.* 2016).

Very recently, Kim *et al.* investigated the expression of PD-L1 in 32 GEP-NETs (stomach (*n*=1), duodenum (*n*=2), biliary tract (*n*=7), pancreas (*n*=14) and 8 hindgut-derived GEP-NETs of distal colon and rectum using IHC. In 7 (21.9%) patients PD-L1 expression was found and this was associated with PFS (PD-L1 positive: 4.2 months and PD-L1 negative: 5.1 months) as well as survival (PD-L1 positive: 16 months and PD-L1 negative: 24.8 months),

indicating a negative outcome for PD-L1 positive GEP-NETs patients (Kim *et al.* 2016).

Cell-based immunotherapy has been addressed in preclinical studies of pheochromocytoma as another example of a neuroendocrine tumor. One peptide of interest was chromogranin A (CgA). Mice with induced pheochromocytoma were vaccinated with CgA. This resulted in larger amounts of infiltrating CD8+ cells and reduction of liver metastases (Papewalis *et al.* 2011). The first studies to test immune checkpoint inhibitors in neuroendocrine tumours are in the process of being started.

Further immune checkpoint receptors

Besides already approved immune checkpoint inhibitors, further molecules are currently under investigation such as T-cell immunoglobulin and mucin domain containing-3 (TIM-3) or lymphocyte activation gene 3 (LAG3). TIM-3 is highly expressed on Tregs, monocytes, macrophages and DCs and is thought to confer reduced antitumor immunity (Zhou *et al.* 2011). Inhibition of TIM-3 in naive CD4+ resulted in proliferation of CD8+ effector cells with increased CD8 T-cell cytotoxicity and increased TH1-mediated interferon- γ release (Sabatos *et al.* 2003, Zhu *et al.* 2005, Boenisch *et al.* 2010). LAG3 is expressed on CD4, CD8 and activated Tregs (Huang *et al.* 2004), B cells (Kisielow *et al.* 2005) and a subset of NK cells (Baixeras *et al.* 1992). LAG3^{-/-} mice have no T-cell defect, but show delay in cell cycle arrest (Workman *et al.* 2004). Interestingly, blockade of LAG3 alone does not reverse the exhausted phenotype of T cells in rodent tumor models, however, combined anti-LAG3/anti-PD-1 antibody therapy has been shown to induce tumor regression (Matsuzaki *et al.* 2010, Woo *et al.* 2012). Further studies on LAG3 and TIM-3 and other potentially interesting immune checkpoint proteins are summarized in an excellent review by Le Mercier *et al.* (2015).

Future perspectives

Endocrine cancers pose several challenges to classical oncologic regimen. One problem is that the proliferation rate and the mutational load are usually very low. Furthermore, due to their rarity and the low number of patients with an aggressive tumor biology, immunotherapy of endocrine malignancy is still at its infancy and clinical trials are mostly lacking. However, not only because of

their higher mutational load, fatal endocrine cancers such as ATC, PDTC, aggressive metastatic MTC and ACC but also G3 neuroendocrine cancers could be suitable tumor entities for immunoncological treatment. It is, therefore, hoped that joint activity of scientists, endocrine cancer networks and patient groups will work toward exploring checkpoint inhibitors, including novel targets such as LAG3 and TIM-3 in combination with other oncological treatments to improve prognosis of patients with aggressive endocrine and neuroendocrine malignancies.

Declaration of interest

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