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REVIEW

The immune tumour microenvironment of neuroendocrine tumours and its implications for immune checkpoint inhibitors

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Abstract

Immunotherapy in the form of immune checkpoint inhibitors (ICIs) has transformed the treatment landscape in numerous types of advanced cancer. However, the majority of patients do not benefit from this treatment modality. Although data are scarce, in general, patients with low-grade neuroendocrine tumours (NETs) do not benefit from treatment with ICIs in contrast to patients with neuroendocrine carcinoma, in which a small subgroup of patients may benefit. Low- and intermediate-grade NETs predominantly lack factors associated with response to ICIs treatment, like immune cell infiltration, and have an immunosuppressive tumour metabolism and microenvironment. In addition, because of its potential influence on the response to ICIs, major interest has been shown in the tryptophan-degrading enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). These enzymes work along the kynurenine pathway that deplete tryptophan in the tumour microenvironment. IDO and TDO are especially of interest in NETs since some tumours produce serotonin but the majority do not, which potentially deplete the precursor tryptophan. In this review, we summarize the current knowledge on the immune tumour microenvironment of neuroendocrine tumours and implications for treatment with immune checkpoint inhibitors. We also discuss (targetable) factors in the NET tumour microenvironment that potentially modulate the anti-cancer immune response.

Key Words

- neuroendocrine tumour
- tumour immune microenvironment
- immune checkpoint inhibitors
- PD-1
- PD-L1
- CTLA-4
- tumour infiltrating lymphocytes
- IDO
- TDO

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Introduction

NETs are tumours that arise from epithelial cells with both neurological and endocrinological functions and are most commonly located in the gastro-intestinal (GI) tract (https://www.cancer.net/cancer-types/neuroendocrine-tumors/introduction). NETs can be divided into two types: symptomatic, due to biogene amine overproduction, or non-symptomatic. The 2017 WHO classification further subdivides NETs into four separate categories: Grade 1, 2 and 3 well-differentiated NETs and grade 3 poorly differentiated neuroendocrine carcinomas (NECs) (Kim *et al.* 2017). Next to this, a study analysing gastric carcinomas found that 10% of these tumours are neuroendocrine malignant tumours and were reclassified as NECs (Waldum *et al.* 1998). The incidence of neuroendocrine tumours in adult patients is 6.98 per 100,000 people, according to the 2012 Surveillance, Epidemiology, and End Results (SEER) data, and this number is increasing (Dasari *et al.* 2017). Median overall survival of all patients with NETs

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is 9.3 years (Dasari *et al.* 2017). However, there is large variation depending on tumour location, stage and grade (Dasari *et al.* 2017). Surgical resection is the only potentially curative option. Palliative treatment options that aim at controlling symptoms and reducing tumour growth are available and include somatostatin analogues, everolimus, sunitinib, peptide receptor radionuclide therapy (PRRT), interferon and chemotherapy. (Pavel *et al.* 2012, Phan *et al.* 2015). The tumour microenvironment (TME) in cancer is recognized as a critical participant in determining tumour biology. Components of the TME include surrounding blood vessels, immune cells, fibroblasts, signalling molecules and the extracellular matrix. In this environment, T cells potentially have the capacity to selectively recognize cancer cells and generate a coordinated immune response. Cancer cells use immune checkpoints to escape recognition by T cells, thereby preventing an adequate anti-tumour immune response. Monoclonal antibodies that target immune checkpoint proteins, including programmed cell death-1 (PD-1, e.g. nivolumab), programmed cell death ligand -1 (PD-L1, e.g. atezolizumab) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, e.g. ipilimumab), can restore the anti-tumour immune response (Herbst *et al.* 2014, Sivan *et al.* 2015, Vetizou *et al.* 2015). Treatment approaches involving CTLA-4 and PD-1/PDL-1 inhibition have successfully improved patient outcomes across various tumour types. However, even in immune-sensitive tumour types, a wide variety of patients do not achieve long-term benefit and factors involved in both primary and secondary resistance have been identified (Blank *et al.* 2016, Chen & Mellman 2017, Seto *et al.* 2019). Clinical data on the effectivity of ICIs in NETs is scarce and the results are inconsistent (Schmidt & Wiedenmann 2018, Weber & Fottner 2018). In this review, we summarize the current knowledge on effectivity of checkpoint inhibition in NETs and discuss (targetable) factors in the NETs TME that potentially modulate the anti-cancer immune response.

Mechanism of action of immune checkpoint inhibitors

ICIs work by interrupting the PD-1 and PDL-1 or CTLA-4 pathway resulting in disinhibition of the tumour evasion mechanisms and thereby enabling T cells to recognize tumour cells and destroy them. Physiologically, PD-1 expression has its role in preventing unnecessary immune responses in peripheral tissues preventing autoimmunity and promoting immune tolerance (Dorfman *et al.* 2006, Raimondi *et al.* 2006). PD-1 is a tyrosine-based inhibitory motif (ITIM)-containing receptor expressed on T cells and interacts with PDL-1 (B7-H1, CD274) and PDL-2 (B7-DC, CD273). Stimulation of the PD-1 receptor typically has greater effects on cytokine production than on cellular proliferation, with significant effects on IFN-γ, TNF-α, and IL-2 production. Interaction of PD-L1 expressed on tumours with PD-1 on immune cells prevents activation of cytotoxic T cells (CTCs) and results in downregulation of cytotoxic cytokine production (Rudd *et al.* 2009). CTLA-4 mediates immunosuppression by reducing signalling through the co-stimulatory receptor CD28 and thereby its interaction with B7 on antigen presenting cells (APC) (Rudd *et al.* 2009). Despite its structural similarity to CD28, CTLA-4 has an opposing effect on T-cell immunity by dampening or actively inhibiting T-cell activation (Rudd *et al.* 2009). CTLA-4 is a homologue of CD28 that has a much higher binding affinity for B7 expressed on antigen presenting cells (APC) but does not produce a stimulatory signal. As such, this competitive binding can prevent the co-stimulatory signal normally provided by CD28:B7 binding. The relative amount of CD28:B7 binding vs CTLA-4:B7 binding determines whether a T cell will undergo activation or anergy (Chambers *et al.* 2001, Greenwald *et al.* 2005).

Treatment with ICIs results in increased progression-free survival (PFS) and overall survival (OS) with subgroups of patients achieving long term survival. For example, in the phase 3 trial (CheckMate 067) in patients with advanced melanoma, the median OS was more than 60.0 months (median not reached) in the nivolumab-plus-ipilimumab group and 36.9 months in the nivolumab group, as compared with 19.9 months in the ipilimumab group. Before the introduction of ICIs, almost all patients died within 2 years of diagnosis (Larkin *et al.* 2019). ICIs have also revolutionised the approach to metastatic NSCLC with single-agent ICIs treatment. ICIs are now the standard of care in the first-line setting in patients with metastatic non-oncogene addicted NSCLC, either alone or in combination with chemotherapy (Vansteenkiste *et al.* 2019). Furthermore, until 2020, the Food and Drug Administration (FDA) has approved ICIs for the treatment of renal cell carcinoma, urothelial and bladder cancer, head and neck squamous-cell carcinoma, metastatic Merkel cell carcinoma, refractory classical Hodgkin lymphoma, microsatellite instability-high cancers (MSI), and gastric cancer (Emens *et al.* 2017, Wolchok *et al.* 2017, Motzer *et al.* 2018, Ward *et al.* 2018, Luchini *et al.* 2019). Despite unprecedented responses, not all patients respond to treatment with ICIs.

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Effectivity of immune checkpoint inhibitors in NET and NEC

Data on response to ICIs in NETs are scarce and inconsistent. KEYNOTE-028 is a single-arm, phase 1b, basket trial that evaluated pembrolizumab (PD-1 inhibitor) in 20 cohorts of patients with a range of advanced solid tumours positive for PD-L1 who had not responded to previous therapies. In this trial, 41 patients with well- or moderately differentiated NETs were included; the primary sites were lung, $n=9$; gut, $n=7$; other, $n=9$; and pancreas, $n=16$. Four patients showed objective responses and 29 patients had at least stable disease for more twhan 6 months (Mehnert *et al.* 2017). Based on these results, the phase II basket trial, KEYNOTE-158, was designed to evaluate pembrolizumab in ten different tumour types, including 107 patients with NETs. NETs originating from pancreas, small intestine, and lung were included. Two-thirds of patients had received at least two prior therapies and 16% expressed PD-L1. After a median follow-up of 18.6 months, the overall response rate was 3.7%, with no complete responses and four partial responses, including three responses in patients with a neuroendocrine tumour of the pancreas and one in a patient with a gastrointestinal NETs (GI NETs). Three of the four patients that responded had a histologically grade 2 NETs. The tumour of one patient was a low-grade pancreatic NETs with aggressively progressive disease at the time of study enrolment. The median PFS was 4.1 months and the 6-month PFS rate was 38%. The median duration of response and median overall survival were not reached, and at 6 months, 85% of patients were alive. Three of the four responses were ongoing after at least 9 months. None of four patients with responses to pembrolizumab had expression of PD-L1 (Ott *et al.* 2019, Strosberg *et al.* 2019).

A small phase II clinical trial including 29 patients with metastatic grade 3 NECs ($n=16$) and moderately differentiated grade 3 NETs ($n=11$) reported on the efficacy and safety of Avelumab (PD-L1 inhibitor) treatment. Site of origin included pancreas ($n=12$), genito-urinary tract ($n=4$), stomach-oesophagus ($n=3$), colo-rectum ($n=3$), lung ($n=2$), ear-nose-throat ($n=2$), papilla of Vater ($n=1$). In responders, mean duration of disease control was 20 weeks, with four patients showing stable disease or partial remission ≥6 months. Median OS was 4.2 months (range 1–12 months) (Fottner *et al.* 2019). Another prospective phase II basket trial (DART) investigating the combination of nivolumab and ipilimumab across 37 subtypes of rare tumours included 33 patients with low-, intermediate- and high-grade NETs and NECs. In the overall cohort of NETs patients, 70% developed progressive disease within 6 months and median OS was at least 11 months. Interestingly, 42% of patients with high-grade NETs and NECs and 0% of patients with low-grade NETs achieved a partial or complete response to treatment (Patel *et al.* 2019). In a phase II clinical trial involving 95 patients with well-differentiated NETs, the primary site was thoracic ($n=30$), pancreatic (pNETs) ($n=33$) and gastro-intestinal ($n=32$) as well as patients with poorly differentiated gastroenteropancreatic NECs (GEP-NECs) ($n=21$). The study analysed spartalizumab, a PD-1 inhibitor, and found that in the well-differentiated cohort, there were seven partial responses (7%) and 55% had stable disease, while 31% had progressive disease. The confirmed objective response rate was 7%, and the disease control rate was 63%. In the GEP-NECs cohort, the objective response rate was 5% and the disease control rate was 19% (Chauhan *et al.* 2018). The previously mentioned studies show that treatment outcomes of ICIs in patients with NETs and NECs are heterogeneous. The NECs studies give signs that response to ICIs is possible. Currently, several studies are ongoing, investigating the value of ICIs for patients with NETs/NECs (Table 1). Now, we will discuss what potential factors are involved in the TME of NETs and NECs that relate to this heterogeneity and irresponsiveness to ICIs.

Hallmarks of response to ICIs and their presence in NETs and NECs

In recent years, several factors that influence response to ICIs have been identified. These include the degree of 'tumour foreignness', T-cell inhibitory mechanisms, the immune cell infiltration and presence of checkpoints of the tumour and other factors in the TME (Blank *et al.* 2016, Chen & Mellman 2017). Here, we describe several of these hallmarks of response specifically focusing on their relevance in NET and their targetability.

Tumour foreignness

Tumour cells are better recognized by the immune system when they are substantially different from normal cells. An altered antigen repertoire presented by the major histocompatibility complex-1 (MHC-1) is influenced by tumour mutational load and allows tumour cells to be recognized by T cells and presented by antigen presenting cells. Mutational load, also known as tumour

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Table 1 Ongoing trials involving ICIs in NETs and NECs.

Therapy	Phase	Patient population	Estimated completion date	NCT number
Ipilimumab + nivolumab	2	GI-cancer/NETs/reproductive neoplasm	Dec 2023	NCT02923934
Nivolumab + ipilimumab	2	NF-GEP or NF-BP NETs	Jan 2024	NCT03420521
Pembrolizumab	2	NECs	Sep 2022	NCT03190213
Pembrolizumab	2	NETs	Jan 2020	NCT02939651
Pembrolizumab	2	G3 NETs or NECs	Dec 2021	NCT03290079
Pembrolizumab + lanreotide	1b/2	GEP-NETs or pNETs	Jun 2020	NCT03043664
Pembrolizumab + chemotherapy	2	BP-NECs	Jun 2020	NCT03135055
Spartalizumab/ PDR001	2	NETs + NECs	Sep 2020	NCT02955069
Pembrolizumab + talabostat mesylate	1b/2	Prostate NETs	Jun 2022	NCT03910660
Avelumab	2	NETs G2/3	Sep 2021	NCT03278379
Avelumab	2	GEP-NECs	Feb 2020	NCT03147404
Avelumab	2	NECs	Jan 2024	NCT03352934
Avelumab	1/2	GEP- or BP-NECs	Sep 2020	NCT03278405
Nivolumab and temozolomide	2	NECs	Dec 2021	NCT03728361
Ipilimumab + nivolumab + cabozantinib S-malate + cabozantinib	2	NECs	Oct 2021	NCT04079712
Durvalumab + tremelumumab	2	G1 + G2 GEP- or BP-NETs and G3 GEP NET	Mar 2020	NCT03095274

BP, bronchopulmonary; G1, grade 1; G2, grade 2; G3, grade 3; GI, gastrointestinal; NCT, clincaltrials.gov identifier; NECs, neuroendocrine carcinomas; NETs, neuroendocrine tumours; NF, non-functioning; pNETs, pancreatic neuroendocrine tumours.

mutational burden (TMB), is defined as the total number of mutations in the DNA of tumour cells. ICIs have changed clinical practice for lung cancer and melanoma, which are tumour types with some of the highest tumour mutational burdens (TMB) (median TMB 7.2 and 13.5 mutations/Mb, respectively). In comparison, a mean TMB of 5.4 was shown for pNETs (Salem *et al.* 2017, Büttner *et al.* 2019). High TMB is likely related to a proportionally higher burden of immunogenic cancer-specific 'neoantigens'; however, these 'neoantigen' proteins must be processed and expressed (van Allen *et al.* 2015, Cogdill *et al.* 2017). RNA-based studies have identified gene expression signatures linked to immune infiltration within the TME that correlate with neoantigen load (Brown *et al.* 2014, Rooney *et al.* 2015). The value of the gene expression signature is that tumours having these mutational epitopes can be identified and a prediction can be made which patients are likely to benefit from checkpoint blockade.

Another way in which the degree of tumour foreignness can increase is through impaired DNA mismatch repair (MMR). Mutations or silencing of genes involved in DNA base pairing results in a DNA chain of altered length and highly repeated sequences (microsatellites), a phenomenon called microsatellite instability (MSI). In May 2017, the FDA approved pembrolizumab for patients with unresectable or metastatic MSI-high (MSI-H) or mismatch repair deficient solid tumours that have progressed following prior treatment (Masuda *et al.* 2011, Le *et al.* 2015). Two studies including 89 small-intestine NETs (siNETs) and 35 pNETs patients analysed DNA MMR and MSI. Both studies showed that, in NETs, defects in DNA MMR were rare and tumours were microsatellite stable (Kidd *et al.* 2005, Arnason *et al.* 2011). A different study investigating DNA MMR and MSI in NECs and mixed adenoneuroendocrine carcinomas (MANECs) included 53 NECs and 36 MANECs patients from several sites of origin. This study demonstrated that 12.4% of patients with either NECs or MANECs were MSI (Sahnane *et al.* 2015). A study comparing well-differentiated NETs ($n=24$) with poorly differentiated NECs ($n=14$) showed that progression of NETs was associated with MSI (Furlan *et al.* 2004). The tumour foreignness of NETs is low, since NETs generally do not differ much from normal neuroendocrine cells. Due to lower E-cadherin levels, neuroendocrine cells may spread and thus metastasize before acquiring multiple mutations and thus have a relatively low degree of tumour foreignness at time of metastatic disease (Waldum *et al.* 2014). These studies show signs that NECs may not develop from normal neuroendocrine cells and this should be further investigated. A subgroup of patients with NENs possess hallmarks that are associated with potential clinical benefit from treatment with ICIs. Higher grade NETs could have a different TME, higher TMB and are more often DNA MMR deficient.

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General immune status and the influence of the microbiome

An impaired general immune status has shown correlations in the effectivity of ICIs due to a reduced ability to mount or maintain a systemic tumour-specific T-cell response (Blank *et al.* 2016). Furthermore, specific compositions of the gut microbiome have been shown to influence the anti-tumour response and response to ICIs. This influence of the gut microbiome on anti-tumour immunity was shown in a preclinical melanoma mouse model. In this study, melanoma growth in mice harbouring distinct commensal microbiota showed a good response to ICIs targeting PD-L1, whereas the other group did not. More interestingly, after faecal cohousing or after faecal transfer of the responding mice into the mice who did not respond, the response to ICIs improved markedly (Sivan *et al.* 2015). The influence of specific gut microbiota on the response to ICIs was confirmed in a clinical study including 112 melanoma patients that were treated with PD-1 targeting ICIs. In this study, it was demonstrated that oral and gut microbiome significantly differed in diversity and composition of responders vs non-responders to ICIs (Gopalakrishnan *et al.* 2018). Interventions manipulating the gut microbiome during immune-based cancer therapeutics aiming to improve response rates are currently in clinical trials. In a study using mice xenografted with the NET cell line BON, germ-free mice were compared to mice colonized with human gut microbiota to identify potential mechanisms through which microbial products such as short-chain fatty acids could augment tryptophan hydroxylase-1 (TPH1) and serotonin synthesis. (Parekh *et al.* 1994, Siddique *et al.* 2009). The study found that gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells (Reigstad *et al.* 2015). Currently, no data on the gut microbiome of patients with NETs and NECs treated with ICIs are available. It is potentially of interest to investigate whether manipulation of the gut microbiome in patients with NETs could lead to a better response to ICIs.

Immune cell infiltration

Immune cell infiltration in the TME is one of the most essential characteristics for an appropriate anti-tumour immune response to develop. Patients with tumours with a high degree of tumour infiltrating lymphocytes (TILs) have a better prognosis and response to ICIs than patients with tumours with a low degree TILs (Harlin *et al.* 2009, Gajewski *et al.* 2013, Gajewski 2015). Important characteristics of TILs are density, distribution and type of TILs. The most favourable profile consists of both CD4 T cells and CD8 T cells because of their complementary role in the anti-tumour immune response. It is commonly accepted that antibody responses against the often poorly immunogenic tumour antigens necessitate strong T cell help and that IL-2 produced by CD4 T cells may be required for growth and proliferation of CD8 T cells. These events have been shown to be necessary for prolonged anti-tumour immunity and complete tumour regression (Gerloni & Zanetti 2005). CD8 T cells can have both effector and memory functions, thereby contributing to therapeutic efficacy of ICIs. (Farhood *et al.* 2019).

The TME of tumours have been classified into 'hot', 'altered (excluded and immunosuppressed)' and 'cold' (Galon & Bruni 2019). The 'hot' immune TME is characterized by a high degree of T-cell and cytotoxic T-cell infiltration (also known as a high Immunoscore) together with checkpoint expression (PD-1, PD-L1 and CTLA-4, T-cell immunoglobulin mucin receptor 3 (TIM3) and lymphocyte activation gene 3 (LAG3) or otherwise impaired T-cell functions. The 'altered-immunosuppressed TME is characterized by poor T-cell- and cytotoxic T-cell infiltration (considered as intermediate Immunoscore), presence of soluble inhibitory mediators, presence of immune suppressive cells and presence of T-cell checkpoints. The 'altered-excluded' immune TME is characterized by no T-cell infiltration inside the tumour bed; accumulation of T cells at tumour borders (considered as intermediate Immunoscore), activation of oncogenic pathways, epigenetic regulation and reprogramming of the TME, aberrant tumour vasculature and/or stroma, and hypoxia. The 'cold' TME is characterized by absence of T cells within and around the tumour edges (also known as low Immunoscore) and failed T-cell priming (low mutational burden, poor antigen presentation and intrinsic insensitivity to T-cell killing) (Galon & Bruni 2019) (Fig. 1).

Tumour infiltration by T cells, NK cells, mast cells, macrophages and dendritic cells in the TME of NETs has been studied. In an observational study investigating 87 patients with NETs, it was found that, in primary intermediate-grade NETs, a dense CD3+T-cell infiltrate was associated with a median recurrence free survival of 128 months compared with 61 months for those with low levels of intratumoural T cells. In the same study, 39 NETs patients with liver metastases (NETLMs) included both low- and intermediate grade primary NETLMs and revealed that the degree of infiltration by CD3+, CD4+ and CD8+ did not predict OS, whereas a low level of infiltrating regulatory T cells (Tregs) was a predictor of prolonged OS

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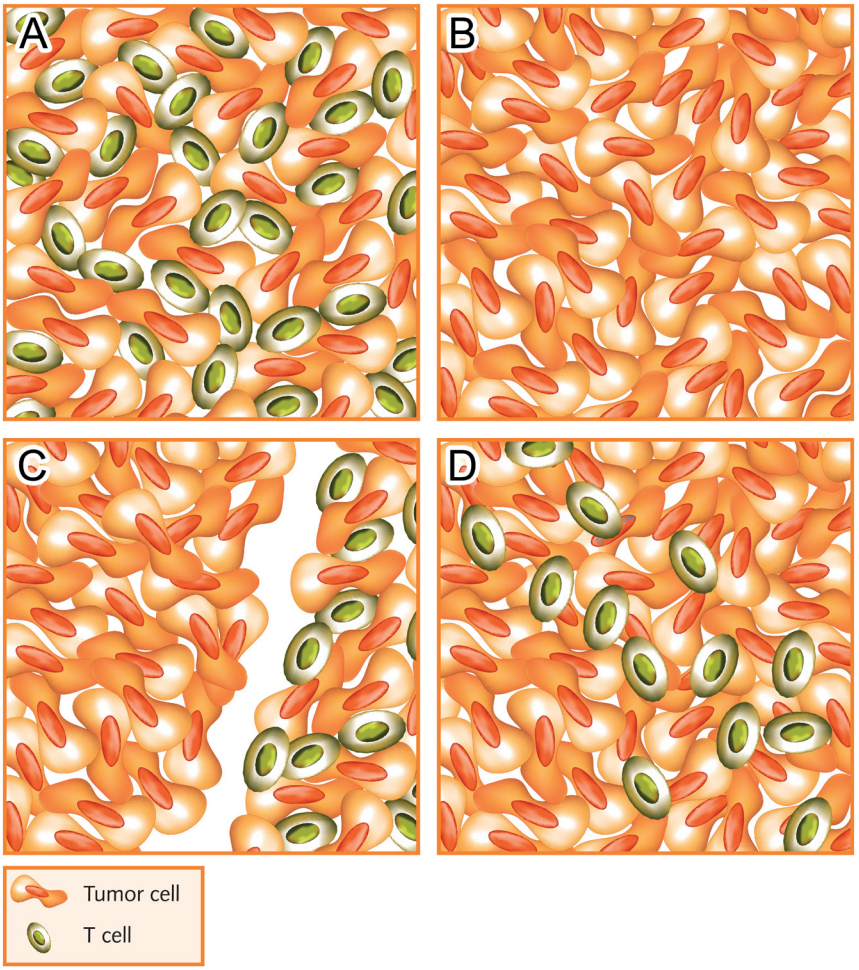

Figure 1
Schematic representation of the immune TME. Four different types of tumour cell infiltration by immune cells like CD3+ and CD8+ T cells can be distinguished. The different types relate to both level and distribution of T-cell infiltration and consist of 'hot' (panel A), 'altered' (can be both immunosuppressed (panel B) or excluded (panel C)) and 'cold' (panel D). These tumour phenotypes are characterized by high (panel A), intermediate (panel B and C) and low Immunoscore (panel D), respectively, which relates to response to immune checkpoint inhibitors.

(Katz *et al.* 2010). In a different study, it was demonstrated that TILs were more abundant in pNETs than in siNETs and that there was no clear association between immune checkpoint marker expression, immune cell infiltrates, and specific mutational profile within each tumour type (da Silva *et al.* 2018). A different retrospective study including 51 patients with grade 1 and 2 NETs analysed the presence of T cells in the immune microenvironment of NETs. The study found that T-cells were present in 15 of the 45 samples, varying between 1 and 10% of T-cells per high power field. T cells were most frequently found within the stroma of NETs of the jejunum/ileum (in 7 of 22 samples), which were all serotonin producing NETs (De Hosson *et al.* 2020).

In a retrospective study in patients with low-grade carcinoid tumours ($n=57$) and patients with high-grade lung NECs ($n=185$), a marked difference in mean CD8+ T-cell infiltration was found (12 vs 92, respectively) (Kasajima *et al.* 2018). Ferrata *et al.* showed that, by combining CD3+ cells and PD-L1 status in patients with grade 3 NETs, they identified the immune ignorant phenotype of tumour microenvironment as being the most common phenotype (Ferrata *et al.* 2019). A retrospective study including 33 patients with NECs of the digestive tract analysed the infiltration in the tumour immune microenvironment and found that $CD3^+$ T-cell infiltration was observed in 23 patients (69.7%), with nine patients detected as high infiltration; $CD8^+$ cytotoxic T-cell infiltration was observed in nine patients (27.3%) and all were detected as low infiltration (Xing *et al.* 2020). The previously mentioned data suggests that the immune TME of lower-grade NETs are infiltrated by T cells but that higher-grade NETs/NECs have an even higher infiltration.

Presence of checkpoints

Tumours express PD-L1 to evade immune mediated killing and PD-L1 expression is, therefore, a logical requirement for response to ICIs treatment. A study of 75 patients with NSCLC treated with a combination of anti-CTLA-4 and anti-PD-1 found that PD-L1 staining is an independent predictor of response (Hellmann *et al.* 2018).

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Another double-blind, phase 3 clinical trial (KEYNOTE 189) involving 616 patients with metastatic non-squamous NSCLC reported that PD-L1 levels may be predictive of response to pembrolizumab plus chemotherapy in the setting of first-line treatment for patients with NSCLC (Gandhi *et al.* 2018). Interestingly, some studies also found that the expression of PD-L1 on particular cells may be an important factor, as PD-L1 can be expressed on both TILs and tumour cells (Tang *et al.* 2018). In independent cohorts of patients with melanoma and patients with urothelial carcinoma, it was found that PD-L1 expression on TILs, but not on tumour cells themselves, was associated with response to anti-PD-1 or anti-PD-L1 treatment (Herbst *et al.* 2014, Rosenberg *et al.* 2016, Mariathasan *et al.* 2018, Havel *et al.* 2019). PD-1 and PD-L1 expression in NETs is more common in poorly differentiates NETs than in well-differentiated NETs. The expression (patterns) of PD-1 and PD-L1 in multiple NETs studies is shown in Table 2 (Kim *et al.* 2016, Roberts *et al.* 2017, da Silva *et al.* 2018, Kasajima *et al.* 2018, Lamarca *et al.* 2018, Wang *et al.* 2018, 2019, Ferrata *et al.* 2019). In general, PD-L1 expression remains an important but imperfect predictor of ICIs response. This imperfection might be due to the fact that different PD-L1 detection assays are used and that there are no standardized criteria for the assessment of PD-L1 positive tumours. Furthermore, even when PD-L1 expression is correlated with response, there are many patients with low to no detectable PD-L1 expression who experience durable clinical benefit (Sunshine & Taube 2015). Therefore, PD-L1 expression itself is not a standalone biomarker for therapeutic decisions in clinical practice for all tumour types. PD-L1 expression appears to be higher in NECs, but its role in prognosticating response to ICIs remains to be elucidated.

Absence of tumour associated macrophages

Tumour inflammation-associated factors can promote tumour progression. The infiltration of immunosuppressive factors in the TME of tumour cells influences the response to ICIs (Mantovani *et al.* 1992, Balkwill & Mantovani *et al.* 2001). Tumour-associated macrophages (TAMs) and their derived cytokines IL-6, TNF, IL-1β and IL-23 are generally recognized as dominant tumour-promoting forces and have a possible influence on the response to ICIs treatment. (Vitale *et al.* 2019) In a study analysing the presence of PD-1 on TAMs in both murine and human models, it was demonstrated that TAMs PD-1 expression negatively correlates with phagocytic potency against tumour cells (Gordon *et al.* 2017).

In a retrospective study in 57 patients with grade 3 NETs, the presence of TAMs was reported to be 59% which coincided with a low CD8+ T-cell infiltration in the TME (Ferrata *et al.* 2019). In accordance with this, a study in 104 patients with pNETs reported a negative correlation between TAMs and CD8+ presence in the TME (Cai *et al.* 2019). Several studies investigating the influence of macrophages in the TME of pNETs concluded that the presence of macrophages in the TME was positively correlated with tumour recurrence risk, higher grade,

Table 2 PD-1 and PD-L1 expression on tumour cells and TILs.

Tumour location	PD-1/PD-L1	Samples	TILs (in %)	Tumour (in %)	Cut-off used for interpretation of positive screening	Metastasis?	Author
GEP NETs	PD-1	120	56	X	Not reported	No	Wang *et al.* 2019
G1 or G2 siNETs	PD-1	70	22.8	X	≥5%	No	Lamarca *et al.* 2018
	PD-L1		24.3	12.8			
pNETs	PD-L1	159	45[a]	45[a]	Tumour: ≥5% TILs: >1%	No	Wang *et al.* 2018
siNETs	PD-L1	64	X	0	≥5%	No	da Silva *et al.* 2018
pNETs		31	X	7.4			
G2 GEP NETs	PD-L1	15	0	X	≥1%	Liver	Kim *et al.* 2016
G3 GEP NECs		17	41				
NECs	PD-1	37	63	16	>1%	No	Roberts *et al.* 2017
	PD-L1		27	14			
BP NETs	PD-L1	57	0	0	≥1%	No	Kasajima *et al.* 2018
BP NECs		185	38	11			
G3 NET	PD-L1	57	24.5	15.7	≥1%	No	Ferrata *et al.* 2019

[a]This study has specified the expression of both TILs and tumour and not separately.
BP, bronchopulmonary; G1, grade 1; G2, grade 2; G3, grade 3; GEP, gastroenteropancreatic; NECs, neuroendocrine carcinomas; NETs, neuroendocrine tumours; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; pNETs, pancreatic neuroendocrine tumours; siNETs, small intestine neuroendocrine tumours; TILs, tumour infiltrating lymphocytes; X, not reported.

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and metastatic disease (Pyonteck *et al.* 2012, Wei *et al.* 2014, Cai *et al.* 2019). These findings support the hypothesis that TAMs play a role in the immunosuppressive TME of NETs by production of cytokines and chemokines.

Due to the abundant presence of TAMs and its possible effect on the immunosuppressive TME in NETs, an interesting target for future therapies might be inhibition of the CD47/signal regulatory protein alpha (SIRPα) axis. This axis is active in both solid and haematological tumour cells which normally helps these tumours evade macrophages (Weiskopf 2017). Normally, CD47 is used by tumour cells to evade recognition by macrophages and, therefore, CD47/SIRPα inhibition enables recognition of tumour cells by macrophages and subsequently activating the innate immune system (don't eat me signal) (Weiskopf 2017).

In NETs patients, somatostatin analogues have widely demonstrated significant improvement in symptomatic relief and tumour control growth by a complex mechanism of action including inhibition of cell survival, angiogenesis and immunomodulation (Alonso-Gordoa *et al.* 2015). Interferon-alpha (IFN-a) had long been established as potential treatment modality of patients with NETs. Already in 1983 it was demonstrated that IFN counteracts the NETs-secreted vasoactive substances (Öberg *et al.* 1983). The efficacy of immunotherapy depends on intact IFN signalling for the promotion of both direct (tumour cell inhibition) and indirect (anti-tumour immune responses) effects (Kline *et al.* 2008). A study analysed gene expression profiles (GEPs) using RNA from baseline tumour samples of pembrolizumab-treated patients with melanoma and demonstrated that immune-related signatures correlate with clinical benefit. The T-cell-inflamed GEP contained IFN-γ-responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance. These features were necessary, but not always sufficient, for clinical benefit of ICIs treatment. The T-cell-inflamed GEPs have been developed into a clinical-grade assay which is also known as IFN-γ signature (Ayers *et al.* 2017). In a more recent study, IFN-γ presence in the TME was shown to be a predictive biomarker for response to ICIs in NSCLC and melanoma (Karachaliou *et al.* 2018). This archival study in 17 patients with NSCLC treated with nivolumab revealed that patients with low IFN-γ in the TME had a median PFS of 2.0 months, whereas patients with high levels of IFN-γ had a PFS of 5.1 months. The same study showed that, in the 21 melanoma patients treated with pembrolizumab, patients with low IFN-γ in the TME had a median PFS of 1.9 months and that this was 5.0 months in patients with a high level of IFN-γ. A phase Ib/II study analysed the combination of pembrolizumab with pegylated-interferon (PEG-IFN) alfa 2b in 43 patients with stage IV melanoma. The study demonstrated that IFN-type 1 (IFN-1) signalling is involved in the anti-tumour immune response in patients with melanoma. At a median follow-up duration of 25 months, the objective response rate was 60.5% and 46.5% of patients had ongoing responses. The median PFS duration was 11.0 months in the whole cohort and median PFS was not reached in patients with a response. The median OS duration was not reached (Davar *et al.* 2018, Romero 2019). A currently recruiting study, including patients with metastasized or unresectable NETs with a low proliferation rate, treats patients with a combination of cyclophosphamide and IFN-a to evaluate whether this treatment regimen decreases the rate of circulatory Tregs (ClinicalTrials.gov Identifier: NCT02838342). In the future, IFN treatment combined with ICIs should be investigated as a combination treatment and its possible benefit in NETs patients.

Absence of inhibitory tumour metabolism

Cancer cells are characterized by reprogrammed metabolism, allowing cancer cells to survive in nutrient poor environments. For example, even in the presence of sufficient oxygen, pyruvate is mainly converted to lactate, a process known as aerobic glycolysis. In both cancer and normal cells, the conversion of pyruvate into lactate takes place due to lactate dehydrogenase (LDH). High serum LDH concentrations correlate strongly with poor response to ICI in melanoma. Furthermore, lactate and low local pH can impair crucial T-cell functions (Blank *et al.* 2016).

The mammalian target of rapamycin (mTOR) signalling pathway has been shown to be a promising target for cancer therapy and is registered for treatment of NETs patients. mTOR is known to have immunosuppressive functions, as it is approved for solid organ transplantation medicine to prevent rejection of the transplanted organ (host vs graft disease). The activated mTOR kinase in a complex with raptor (mTORC1) leads to the phosphorylation of ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), two key proteins that regulate protein translation of several proteins necessary for cellular proliferation and growth that have shown antitumor activity in two phase 2 studies involving patients with pNETs (Yao *et al.* 2008, 2010). Both rapamycin and everolimus bind immunophilin FK506-binding protein 12 and inhibit mTOR signalling (Moreno *et al.* 2008). A phase 3 clinical trial in 410 patients with

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advanced low-grade or intermediate-grade pNETs analysed the effect of everolimus in comparison with placebo (Yao *et al.* 2011). This study demonstrated that median PFS was 11 months in patients receiving everolimus and 4.6 moths in patients receiving placebo. Estimates of the proportion of patients who were alive and progression-free at 18 months was 34% with everolimus as compared with 9% with placebo. In a recent pre-clinical study with mice that have renal cell carcinoma, the combination of anti-PD-L1 ICIs and everolimus was investigated. The study found that the combination of everolimus with anti-PD-L1 ICIs significantly reduced tumour burden compared with the everolimus alone treatment, increasing TILs and the ratio of cytotoxic $CD8^+$ T cells to TILs (Hirayama *et al.* 2016).

In cancer cells, >95% of tryptophan is catabolized via the kynurenine pathway by IDO, generating kynurenines (Pschowski *et al.* 2017). Kynurenines are known for their suppression of T cells and induction of apoptosis of T cells. Overall, IDO-induced tryptophan depletion might trigger an immunosuppressive TME in tumours via depletion, anergy, and apoptosis of T cells. Thus, IDO-induction plays an important role in the development of immunological tolerance and IDO-induced immunosuppression is used by solid malignancies to protect themselves from immune recognition and cytotoxicity – a fact that is recognized as a key tumour escape mechanism (Moffett & Namboodiri 2003, Puccetti & Grohmann 2007, Platten *et al.* 2012, Pschowski *et al.* 2017). Remarkably, in a phase 3 study analysing the effectivity of pembrolizumab and epacadostat (IDO-inhibitor) in patients with melanoma, it was demonstrated that there was no benefit in terms of PFS or OS (Long *et al.* 2018).

An interesting study performed by Opitz *et al.* shows that TDO is also frequently activated in cancer, predominantly when IDO is not activated (Opitz *et al.* 2011). The tryptophan-to-kynurenine metabolic pathway in tumour cells also uses TDO for the generation of kynurenine (Wardhani *et al.* 2019). TDO itself plays a role in cancer-cell migration which is a characteristic of TDO which IDO has never been shown to do (Prendergast 2011). This points towards functional differences between the enzymes even though both being able to generate kynurenine. Kynurenine is reported to be an endogenous ligand of the aryl hydrocarbon receptor (AHR) which plays a role in the signalling pathway of TDO to AHR that promotes malignant growth of the tumour. Kynurenine was also demonstrated to play a role in the generation of Tregs. Due to the role of Tregs in the immunosuppressive TME of tumours, kynurenines contribute to immune evasion by binding to AHR (Prendergast *et al.* 2010).

Next to the function of AHR in Tregs, AHR also negatively influences the immunogenicity of dendritic cells which is an important influential factor in the modulation of inflammation and immunity, therefore, suggesting the influence of IDO/TDO-kynurenine-AHR signalling pathway in the microenvironment of tumours (Gajewski *et al.* 2006, Cogdill *et al.* 2017, Havel *et al.* 2019, Heidegger *et al.* 2019). Due to the effects of IDO and TDO in cancer cells, an interesting therapeutic option in the future might be the use of an IDO/TDO dual inhibitor in combination with ICIs. A phase 1 study is currently investigating the safety and tolerability of an IDO1/TDO dual inhibitor in 30 patients with advanced solid tumours (ClinicalTrials.gov Identifier: NCT03208959). This might also be a promising combination with ICIs in NETs.

The reason for the immunosuppressive TME in serotonin-producing NETs is that they use tryptophan for the secretion of serotonin. This can lead to the utilization of 60% of the tryptophan pool for serotonin synthesis in the tumour (Fleischmajer & Hyman 1961, Castiello & Lynch 1972, Bender 1983). This derangement may then result in tryptophan depletion (Bouma *et al.* 2016). T cells activated under tryptophan-deficient conditions are able to synthesize protein, enter the cell cycle, and progress normally through the initial stages of G1, including upregulation of IL-2 receptor and synthesis of IL-2. However, in the absence of tryptophan, cell-cycle progression is halted at a mid-G1 arrest point, thereby diminishing T cell proliferation (Munn *et al.* 1999). Both the secretion of serotonin and generation of kynurenines lead to tryptophan depletion which in itself creates an immunosuppressive TME in NETs. Furthermore, since IDO and TDO are both active in NETs, higher levels of kynurenine also lead to an immunosuppressive TME and these pathways likely influence the TME in NETs (Fig. 2).

Discussion and future directives

NETs and NECs are tumours that can originate anywhere in the body. A growing amount of available data on the TME of these tumours creates new therapeutic opportunities. Immunotherapy has changed cancer treatment approaches in several tumour types and efforts are ongoing to explore the efficacy of immune checkpoint inhibitors in patients with NETs and NECs. Expression of PD-L1, lymphocyte infiltration, mismatch repair deficiency, tumour mutational load, and neoantigen load are predictors of response to immune checkpoint blockade. NETs generally exhibit a 'cold' tumour immune

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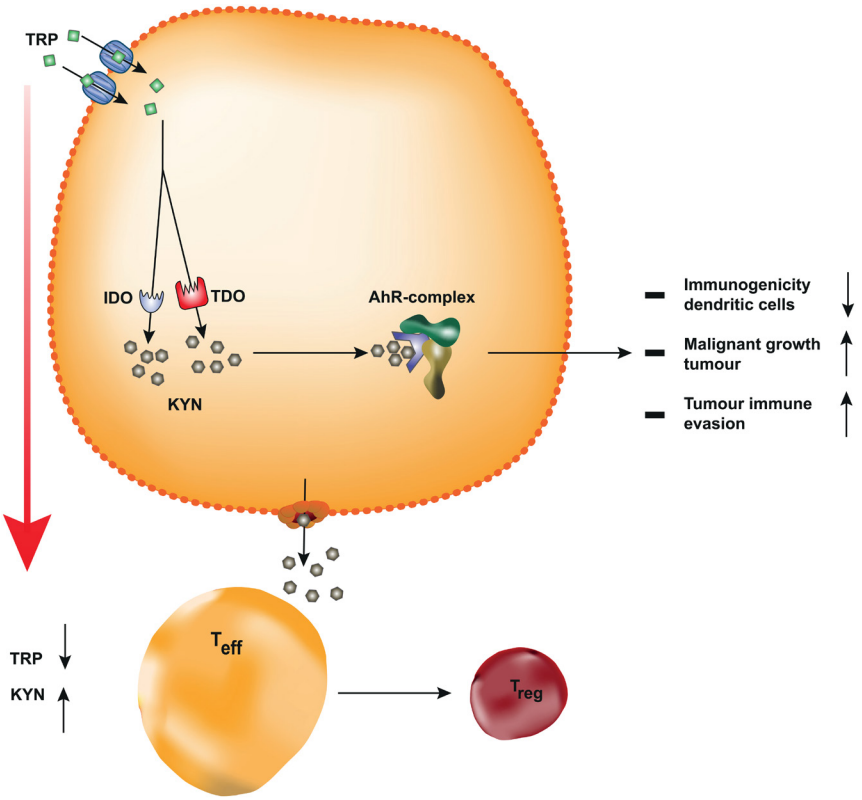

Figure 2
Schematic representation of the IDO/TDO pathway and its influence on the TME of NETs and NECs. IDO and TDO catalyze the conversion of tryptophan into its derivative kynurenine. As a consequence, tryptophan depletion triggers amino acid-deprivation-associated apoptosis of effector T cells. Accumulated kynurenine acts as a ligand for the aryl hydrocarbon receptor (AhR). In a manner that is dependent on AhR, kynurenine promotes the regulatory T-cell phenotype, further contributing to the suppression of antitumor immune responses. Finally, kynurenine potentiates autocrine signaling through AhR on cancer cells themselves, promoting degradation of the extracellular matrix and invasion (Pavlova cell metabolism).

microenvironment lacking several of these favourable factors. Lymphocyte infiltration is often seen in NETs, but it is unclear whether TILs are effectively primed by tumour neoantigens, given the relatively low proportion of cases positive for PD-L1. Most NETs appear to be mismatch repair proficient, and the mutational burden of such malignancies is relatively low compared to NSCLC and melanoma. In contrast, given their extensive mutational load and denser immune infiltration, NECs are likely more suitable targets for immunotherapy (Cives *et al.* 2019).

Several pre-clinical and clinical studies have investigated the response to several types of ICIs in both NETs and NECs. Based on these limited data, we know that patients with NETs do not show robust responses to immune checkpoint blockade antibodies (Mehnert *et al.* 2017, Ott *et al.* 2019, Strosberg *et al.* 2019). Only a small part of patients with NET respond to ICIs. In particular, patients with higher grade NETs and NECs showed better responses ranging from 7 to 42% (Chauhan *et al.* 2018, Fottner *et al.* 2019, Patel *et al.* 2019).

The expression of IDO and TDO in a proportion of patients with NETs may be one of the mechanisms responsible for this 'cold' immune microenvironment. Especially serotonin-producing NETs express IDO and TDO. These patients often have low tryptophan levels, since tryptophan is the sole precursor of both peripherally and centrally produced serotonin. Furthermore, IDO catalyses tryptophan into kynurenines and thereby creates an immunosuppressive TME. This suggests that IDO-mediated immune suppression is most prominent in patients with low tryptophan levels and that these patients might therefore be interesting candidates for treatment with ICIs combined with IDO inhibitors. TDO catalyses the conversion of tryptophan into its derivative kynurenine. Tryptophan depletion triggers amino acid-deprivation-associated apoptosis of effector T cells. Accumulated kynurenine acts as a ligand for AhR. In a manner that is dependent on AhR, kynurenine promotes the regulatory T-cell phenotype, further contributing to the suppression of antitumor immune responses. TDO inhibitors might therefore be an interesting therapeutic option to decrease the immunosuppressive TME of NETs because inhibition of TDO results in decreased kynurenine, resulting in immune activation. Due to the abundant presence of TAMs, and their inverse relation to T-cell infiltration in the TME of NETs, combination of CD47 inhibitors with ICIs might be an interesting option for investigation in future studies. An analysis in 22 refractory B-cell non-Hodgkin lymphoma (NHL) patients demonstrated an objective 50% response and a complete response of 36% when combining ICIs that inhibit CD47 and an antibody inhibiting CD20 (Advani *et al.* 2018).

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Although the overall presence of PD-L1 is low in tumours of patients with NETs, the presence of PD-L1 does not necessarily correlate with response to ICIs. The same has been shown in melanoma and NSCLC, therefore, the focus should be taken off PD-L1 as a standalone biomarker for therapeutic decisions in clinical practice. In future studies, the value of PD-1, CTLA-4 and other potential biomarkers such as, for example, the microbiome composition should be further explored.

Conclusion

In conclusion, immunotherapy and, in particular, ICIs have transformed treatment of several types of cancer. NETs responses to ICI in clinical trials have overall been disappointing. In this review, we presented several key aspects of the TME in NETs which may influence response rate to ICIs. These include a low infiltration of CD8+ T cells in NETs and a high infiltration of immunosuppressive cells such as Tregs and macrophages which results in an immunosuppressive TME. Possible reasons for this immunosuppressive TME are the low concentration of IFN and the production of serotonin, IDO and TDO and the subsequent production of kynurenines. This also presents us with possible combinational treatment options incorporating inhibitors of these factors together with ICIs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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