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Review

The tumor immune microenvironment in gastroenteropancreatic neuroendocrine neoplasms

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ABSTRACT

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are a group of rare tumors that are increasing in prevalence. The complex tumor immune microenvironment (TIME) plays an important role in tumor development and the response to immunotherapy but is poorly understood. In this review, the components of the TIME are described in detail, including discussion about infiltrating immune cells, the immune checkpoint system, the cytokine and chemokine milieu, and immunomodulatory factors. Moreover, a comparison between TIMEs among different types of GEP-NENs and the interplay among the TIME, tumor cells, and the stromal microenvironment is described. Novel treatment options for GEP-NENs and potential biomarkers for the immune response are also characterized. We provide a comprehensive generalized review of the TIME that can inform GEP-NEN treatment strategies.

1. Introduction

Neuroendocrine neoplasms (NENs) are a collection of rare, heterogeneous tumors that originate from the diffuse neuroendocrine system. NENs are defined as epithelial tumors with neuroendocrine differentiation [1]. Although NENs can occur in almost all organs [2], gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are the most common type, and account for 54.5% of all NEN cases [3]. Notably, GEP-NEN incidence has been increasing over the past four decades [4,5]. The 2017 WHO classification for NENs divides GEP-NENs into four categories, as follows: Grade 1 or 2 well-differentiated neuroendocrine tumors (NETs), Grade 3 poorly differentiated neuroendocrine carcinomas, and Grade 3 well-differentiated NETs [6–8]. Over the past several decades, considerable progress has been made in the GEP-NEN field including several treatment strategies that have had significant clinical effects and the identification of novel predictive biomarkers for diagnosis and prognosis. However, novel therapeutic efforts that would further improve clinical outcomes have been constrained by the heterogeneity and limited biological characterization of GEP-NENs. Most of the current studies describing GEP-NENs are retrospective and a systematic review of the tumor immune microenvironment (TIME) is extremely rare. Recently, genomic studies of tumor cells have contributed to the biological understanding of GEP-NENs, including a description of the whole-genome pancreatic neuroendocrine neoplasm (Pan-NEN) landscape. In addition, the genetics and epigenetics of GEP-NENs were recently summarized [9,10]. Tumor growth and progression requires an optimal microenvironment, which consists of various cell types and stromal components including fibroblasts, multiple types of infiltrating immune cells, a distinct cytokine and chemokine milieu, collagen, and other components. Despite the critical importance of the microenvironment to tumor growth, few reviews describe the NET tumor microenvironment, and the role of the TIME in GEP-NENs remains ambiguous [11].

Most studies explore the clinical impact or prognostic value of a single immune cell type or a specific molecule with immunohistochemistry analyses. Infiltrating immune cells, including members from both the innate and adaptive immune system have been studied, including tumor-associated macrophages (TAMs), tumor-infiltrating lymphocytes (TILs) such as $CD8^+$ cytotoxic T lymphocytes (CTLs), regulatory T cells (Tregs), and B cells, tumor-infiltrating neutrophils (TINs), natural killer (NK) cells, mast cells, and dendritic cells (DCs) [12]. Components of the immune checkpoint system including

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the programmed death-1 (PD-1) receptor and its ligands, programmed death ligand-1 (PD-L1) and PD-L2, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), have also been investigated in detail. Finally, the cytokine and chemokine milieu including tumor necrosis factor-α (TNF-α), IL-2, transforming growth factor β (TGF-β), C-X-C motif chemokine receptor 4 (CXCR4), vascular endothelial growth factor (VEGF)-A, and interferon (IFN), as well as immunomodulatory factors including CD73, CD133, CD166, and CD56 have also been studied [13]. These studies identify critical TIME components and illustrate their clinical significance in GEP-NENs. However, a comprehensive analysis of the crosstalk among these components within TIME does not exist and would be a valuable resource for clinical research efforts. The treatment options for advanced, nonresectable patients are limited, and the identification of novel therapeutic targets that advance current treatment strategies is a crucial unmet need for GEP-NENs.

In this review, we summarize the infiltrating immune cells, the immune checkpoint system, the cytokine and chemokine milieu, and the immunomodulatory factors within GEP-NENs. We also compare the TIME among different types of GEP-NENs and illustrate the interplay among the TIME, tumor cells, and the stromal environment. Finally, we summarize novel treatment options related to the TIME for GEP-NENs. This review presents the complex framework of the TIME that exists in GEP-NENs and provides a comprehensive, guiding reference for future research.

2. TIME of GEP-NENs

Due to the data scarcity and the distinct biological characteristics of G3 NECs, this type of disease is often discussed separately. Research describing the TIME of GEP-NENs is increasing, and a complete overview of the field is necessary (Fig. 1).

2.1. Infiltrating immune cells in GEP-NETs

Immune cell infiltration, including TAMs, T cells, B cells, TINs, NK cells, mast cells, and DCs, has been reported in GEP-NENs.

Macrophages generally have two possible functional states: classically activated type 1 macrophages (M1) that produce high levels of pro-inflammatory cytokines and promote the immune response and alternatively activated type 2 macrophages (M2) that suppress Th1-mediated inflammation by secreting IL-10 and IL-1b and promote tumor growth through secretion of a series of cytokines [14]. TAMs are thought to be a paradigm for M2 macrophages [15]; however, the M1/M2 theory is over-simplified and ignores the possible continuum of intermediate phenotypes of macrophages. Furthermore, Pollard et al. suggested that M1/M2 polarization does not exist in the TIME and that a unique TAM population was educated by the tumor microenvironment to carry out tumor-promoting functions [16]. Therefore, we have restricted our discussion to the role of TAMs, which are characterized by their protumoral role and are the most informative prognostic factor within the TIME [17,18]. In this context, TAMs in Pan-NENs are correlated with higher tumor grades, higher cancer stages, liver metastases, increased recurrence risk, and shorter disease-free survival [19–21]. TAMs are involved in creating an immunosuppressive TIME by releasing cytokines and chemokines and by promoting the release of inhibitory checkpoint proteins in T cells, thereby providing crucial functions for regulating tumor progression and metastases [22,23].

Infiltrating T cells have been studied in GEP-NENs. da Silva and colleagues assessed T-cell infiltration in the intra and extratumoral areas [24]. General T cells were identified by CD3, memory T cells were identified by CD45RO, and CTLs were identified by CD8. All three cell types were expressed at similar levels in the intratumoral compartment in both Pan-NENs and small intestine neuroendocrine neoplasms (SI-NENs). However, in the extratumoral area, Pan-NENs exhibited increased infiltration of all three T-cell subsets when compared to

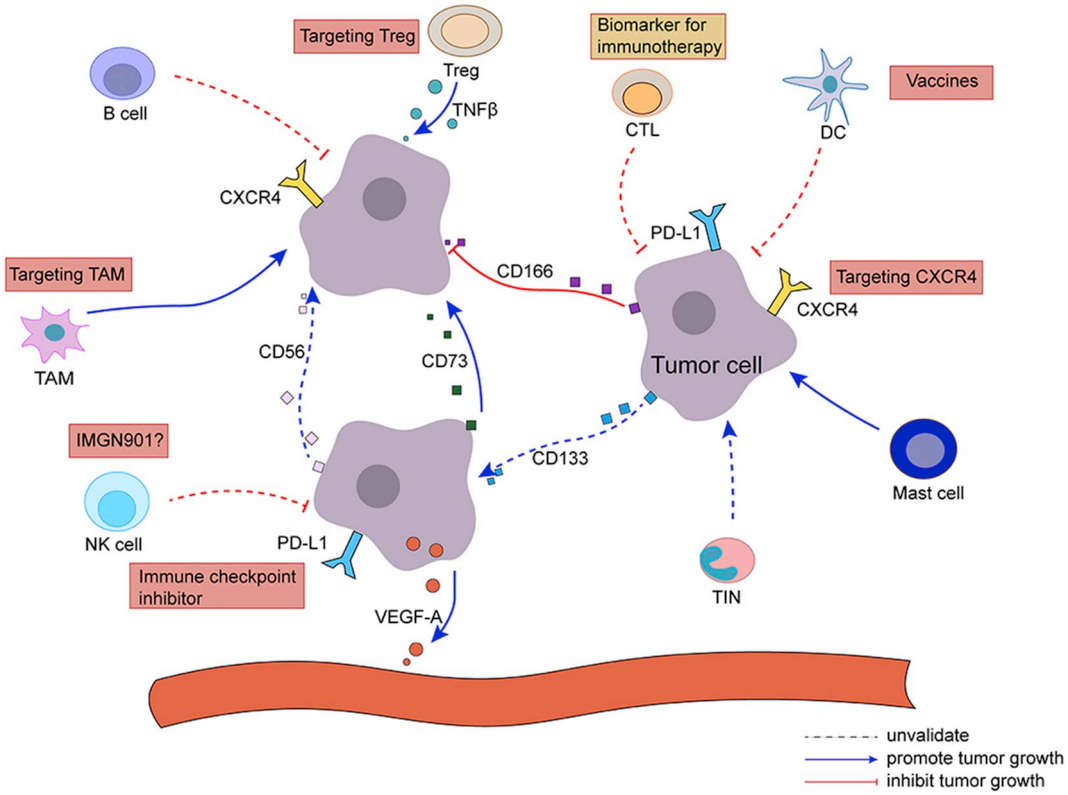

Fig. 1. The TIME components of GEP-NENs and their clinical significance. TAM, tumor-associated macrophages; CTL, cytotoxic T lymphocytes; Treg, regulatory T cells; TIN, tumor-infiltrating neutrophils; DC, dendritic cells; TGF-β, transforming growth factor-β; VEGF-A, vascular endothelial growth factor-A; TIME, tumor immune microenvironment; GEP-NENs, gastroenteropancreatic neuroendocrine neoplasms.

SINENs. These results indicate a more robust immune response in Pan-NENs when compared to SINENs. Moreover, duodenal NENs exhibit a higher immune infiltration than jejunal or ileal NENs [25].

A prior study demonstrated that a dense $CD3^+$ T-cell infiltration was predictive of prolonged, recurrence-free survival (RFS) in intermediate Pan-NENs and liver metastases after resection. $CD4^+$ and $CD8^+$ T-cell infiltration is not statistically associated with improved RFS [26]. In contrast, a recently published study that included immunohistochemistry analyses of 244 GEP-NEN cases reported that high expression of TILs is associated with significantly shorter survival and higher tumor grades. These findings suggest immune evasion by tumors [27].

Tregs, which are identified by FOXP3, play a crucial role in tumor immune escape and contribute to an immunosuppressive microenvironment by secreting TGF-β and IL-10. Importantly, Tregs are also positively correlated with GEP-NEN grade, as classified by the WHO [28,29]. Because increased Treg density is significantly correlated with poor overall survival (OS), it could be an independent prognostic factor for patients with Pan-NENs and liver metastases [26]. In peripheral blood, circulating Tregs are elevated in patients with midgut carcinoid when compared to normal controls [30]. The ratio of CTLs to Tregs is reported to be a better prognostic indicator than a single lymphocyte subtype. Indeed, the infiltration of immune cells in 19 cancer types with a gene-based computational method indicated that the CTLs-to-Tregs ratio was correlated with improved survival [31]. The potential of the CTL-to-Treg ratio to be used as a prognostic factor in GEP-NENs warrants further research.

Although T cells have been reported in GEP-NENs, studies that specifically focus on the role of B cells are sparse, and more research is required to overcome this knowledge gap. TINs in GEP-NENs are poorly understood and existing studies have primarily focused on the prognostic significance of the neutrophil-to-lymphocyte ratio (NLR) in the peripheral blood [32–36]. An elevated preoperative NLR is a prognostic factor that can potentially be used to identify GEP-NENs with poor outcomes. Huang and colleagues reported the predictive value of the tumor-infiltrating NLR, and found that an increased NLR was significantly correlated with tumor progression and metastasis in gastric NENs [35]. The effect of infiltrating NK cells in GEP-NENs is not well understood. Since the discovery of NK cells in the 1970s, many studies have shown that NK cells play a pivotal role in antitumor cell cytotoxicity. One study, in the 1990s, indicated that NK cell activity within gastrointestinal (GI) NENs is modulated by hormones and that increased NK cell activity is related to tumor regression [37]. In addition, another study reported a deficient interferon (IFN) response in patients with midgut carcinoids and that the NK cell activities in these patients could be restored by in vitro IFN exposure [38]. Soucek and colleagues explored the association between mast cells and Pan-NENs. They reported that rapid mast cell recruitment to tumor sites was necessary for tumor progression. The inhibition of mast cell degranulation was favorable in Pan-NENs [39,40]. In the past five decades, since the first discovery of DCs in the late 1970s, DCs have been established as professional antigen-presenting cells that specialize in activating naive and memory $CD4^+$ and $CD8^+$ T cells. Indeed, several studies now show that increased infiltration of DCs within the TIME is associated with better clinical outcomes [41,42]. However, the study by Katsenelson and colleagues identified that DCs were absent in bronchial carcinoid tumors and found that soluble factors produced by lung tumors can inhibit DC maturation or generation [43]. In Pan-NENs, it is reported that a frequent loss of beta-2 microglobulin hinders DC antigen presentation [44]. Overall, the literature indicates that DCs may be absent or dysfunctional in GEP-NENs.

Another relevant immune player, tertiary lymphoid structures (TLS), which are ectopic lymphoid organs that reflect lymphoid neogenesis, are found in tumors where they orchestrate adaptive antitumor responses [45]. TLS were found in 18% of SINENs; however, their biological significance remains unclear [25].

2.2. Infiltrating immune cells in GEP-NECs

NECs greatly differ from NETs with respect to histology, morphology, proliferation rate, and clinical characteristics. New evidence has shown that NECs are a distinct clinical entity [12]. Compared to GEP-NETs, GEP-NECs have lower incidence numbers and account for < 1% of all gastrointestinal carcinomas [46]. Pan-NECs have more frequent mutations in p53 and RB, which may account for some of the differences between the Pan-NEC TIME and the Pan-NET TIME. There is a prevailing hypothesis that GEP-NECs have a distinct TIME when compared to GEP-NETs.

The TIME could be described as “hot” if the tumor is highly infiltrated by immune cells or as “cold” if the tumor is devoid of immune cells. Tumors with a hot TIME may respond to treatment by immunotherapeutic strategies, whereas tumors with a cold TIME are often unresponsive [47]. Takahashi and colleagues used multiplexed fluorescent immunohistochemistry to reveal that Pan-NECs had a hot TIME that was characterized by a large number of TILs [48]. Notably, the cells that infiltrated in the greatest numbers in Pan-NECs were $CD3^+CD4^+$ T cells. $CD20^+$ TILs were also abundantly expressed. However, the hot TIME of Pan-NECs did not show any beneficial impact on survival and its biological function is not currently understood.

2.3. Immune checkpoint system in GEP-NENs

The immune checkpoint receptor PD-1 and its ligands PD-L1/PD-L2 play a critical role in the formation of an immunosuppressive microenvironment. PD-1 is rarely expressed in either Pan-NENs or SINENs. On the other hand, PD-L1 is expressed in 9.56–30% of all GEP-NENs [49–52]. However, the intratumoral expression of PD-L1 is complex and massive heterogeneity in expression patterns exist between Pan-NENs and SINENs. Indeed, 97% of Pan-NENs expressed PD-L1, but it was not expressed in SINENs [24]. Moreover, PD-L1 expression was particularly elevated in high-grade NENs, GEP-NECs, and poorly differentiated tumors [53]. Intriguingly, the cytoplasmic expression of PD-L2 was also observed in a high proportion of tumor cells including 82% of SINENs and 97% of Pan-NENs.

The expression of PD-1 and PD-L1 is correlated with decreased survival and increased tumor grade in GEP-NENs [27]. Similarly, the expression of PD-L1 is also associated with decreased progression-free survival and OS in patients with metastatic GEP-NENs [51]. In contrast, a recent study with SINEN patients reported that PD-L1 expression does not have any impact on OS or progression-free survival. However, these contradictory results may be partially explained by the small sample size [52]. Overall, higher expression of PD-1/PD-L1 is associated with a more robust response to immunotherapies, which include anti-PD-1/PD-L1 drugs. This indicates that PD-1/PD-L1 could be both a prognostic factor and a predictive factor for immunotherapy efficacy.

Significantly less is known about the role of CTLA-4 in GEP-NENs. A single study reported on a patient with metastatic functional G2 gastric NEN. The patient had an extended survival after a combination of PD-1 and CTLA-4 blockade therapeutics. These data indicate that interrupting CTLA-4 could be a potential treatment for GEP-NENs, but more exploration and an expanded patient cohort are needed to fully evaluate the therapeutic potential [54].

2.4. Cytokine and chemokine milieu of GEP-NENs

Data describing the cytokine and chemokine milieu of GEP-NENs are accumulating (Fig. 1). However, fundamental questions remain, including: Which factors are present in the tumor microenvironment? What is the interplay between these factors and immune cells? Which factors are associated with a poor prognosis? How does the milieu evolve during the process of tumor progression and metastases? Completely understanding the function of cytokines and chemokines within the TIME will provide new opportunities for developing innovative

cancer immunotherapies.

TNF-α and IL-2 are associated with the development of GEP-NENs. Specifically, high IL-2 serum levels may distinguish functional and non-functional GEP-NENs [13]. Similarly, elevated IL-6 serum levels could be an important biomarker of non-functional Pan-NENs.

TGF-β is a pleiotropic polypeptide produced by many cell types, and this factor plays an essential role in preventing intestinal inflammation [55]. As a suppressor of immunosurveillance in cancer, TGF-β has been hypothesized to impair the protective immune response of GEP-NENs [56]. A prior study explored the interplay between the TGF-β and somatostatin signaling pathways in the human NET cell line, BON. Notably, TGF-β had antiproliferative effects that occurred through both c-Myc downregulation and somatostatin signaling. Indeed, disruption of the link between TGF-β and somatostatin signaling is associated with increased metastatic potential where TGF-β becomes a growth accelerator instead of a growth inhibitor [57]. The inhibitory effects of TGF-β on tumor growth and metastasis in GEP-NENs depends on the connectivity between TGF-β and the somatostatin signaling pathway.

CXCR4 is expressed in various cancers including GEP-NENs. The chemokine receptor plays a crucial role in leukocyte recruitment and is also important during embryogenesis [58]. Exploring the expression of CXCR4 in patients with GEP-NENs using [68Ga]-Pentixafor-PET/CT revealed that positive-CXCR4 was primarily expressed in aggressive GEP-NECs (Ki67 ≥ 85%). Interestingly, low-grade tumors did not express the receptor [59]. Furthermore, an inverse expression pattern between the CXCR4 and somatostatin receptor (SSTR) was also identified in GEP-NENs: As tumor grade increased, CXCR4 expression increased and SSTR 2A decreased. Accordingly, most G3 GEP-NECs are CXCR4-positive and SSTR-negative [60]. The prevailing treatment for NECs is platinum-based chemotherapy and after first-line chemotherapy failure, the treatment options are limited. Considering the robust expression of CXCR4 in patients with NECs, CXCR4 may be a potential therapeutic target. Large-scale studies are warranted to comprehensively evaluate the prognostic value and the treatment potential of CXCR4 in GEP-NENs.

GEP-NENs are characterized by a high vascular supply. Importantly, VEGF-A is a major cytokine associated with tumor-associated neovascularization, which can suppress the beneficial effects of T cells and inhibit DC maturation [61]. In the study by Cigrovski and colleagues, strong VEGF-A expression was observed in low-grade Pan-NENs and was correlated with a good prognosis. Similar conclusions were made in GI-NENs: Tumors with low VEGF expression were more invasive when compared to tumors with high VEGF expression. Furthermore, a negative association between VEGF and Ki67 was found in 145 GEP-NEN cases [62]. Single nucleotide polymorphisms in VEGF-A and the VEGF receptor-3 were also associated with a poor prognosis in GEP-NENs. Therefore, VEGF and its associated receptors are potential prognostic factors that could guide the clinical management of GEP-NENs [63]. In summary, VEGF is a favorable prognosis biomarker while single nucleotide polymorphisms of VEGF are an unfavorable biomarker for GEP-NENs.

IFN-α was first introduced in the clinic to treat midgut carcinoids in the 1980s and had encouraging results. This cytokine controlled hormone hypersecretion, tumor growth, and angiogenesis by inhibiting cell cycle arrest and VEGF gene transcription [64]. Currently, IFN-β is thought to have more pronounced effects than IFN-α, although both cytokines exhibit potent antitumor effects in NENs cells [65,66]. In addition, the recently discovered IFN-λ family, including IL-28A/B and IL-29, has the same antiproliferative effects as IFN-α and IFN-β [67]. Moreover, a prospective trial demonstrated comparable antiproliferative effects between somatostatin analogs (SSA), IFN-α, or a combination of the two in metastatic GEP-NEN treatment [68]. It is important to note that the antiproliferative effects of these treatments are often limited by IFN resistance. A study by Zitzmann and colleagues revealed an important role for suppressor of cytokine signaling protein 1 (SOCS1) in IFN resistance. SOCS1 knockdown in NEN cells was a promising strategy to increase the efficacy of IFN treatment in GEP-NENs [69]. Despite the clinical promise of IFNs as therapeutics, they are not used as a first-line therapy due to unfavorable toxicity. In summary, IFN is a promising ancillary treatment for GEP-NENs that has antiproliferative effects on tumor cells.

High expression of the genes that encode the chemokines CX3CL1, CXCL9, and CXCL10 is correlated with infiltration of T cells within the TIME, prolonged disease-free survival, and OS in colorectal cancer [70]. In addition, the gene expression of several chemokines including CCL19, CCL21, CCL17, CCL22, CXCL13, and IL-16 is associated with the recruitment of T cells from the blood into the tumor microenvironment in lung cancer [71]. Chemokines can attract different immune cells into the TIME, and immune cells can express receptors for a diverse array of chemokines. Therefore, the cytokine and chemokine milieu of GEP-NENs and its interaction with immune cells in GEP-NENs warrant further investigation.

2.5. Immunomodulatory factors in GEP-NENs

The cluster of differentiation, which is commonly used to classify immune cells on the basis of cell-specific markers can also be used to understand the tumor immunomodulatory response.

Ecto-5′-nucleotidase (CD73), converts adenosine monophosphate into adenosine, which is a crucial molecule for tumor immune tolerance [72]. In addition, CD73 also exhibits nonenzymatic functions that promote cancer progression by regulating the interaction between tumors and extracellular matrix components [73]. Notably, CD73 was expressed on the cell membranes of 27.2% of GI-NENs. A higher positive ratio of PD-L1 was also found in the CD73-positive group when compared to the CD73-negative group, which suggests that CD73 expression status is closely related to PD-L1 expression in GI-NENs [49]. CD73 is frequently overexpressed in several types of human cancer where is has an important role in tumor angiogenesis, growth, and metastasis. Multiple studies support the premise that CD73 expression is associated with a poor prognosis, and this trend holds for a variety of tumor types including triple negative breast cancer, malignant melanoma, gastric cancer, colorectal cancer, gallbladder cancer, and serous ovarian cancer [74]. Additionally, immunohistochemical staining revealed that increased CD73 expression was associated with invasion into adjacent organs, which further indicates that CD73 may be an unfavorable prognostic factor for Pan-NENs [75].

Prominin-1 (CD133) is a five transmembrane single-chain glycoprotein that is widely accepted as a stem cell marker and is expressed in numerous types of solid tumors [76]. It was reported that CD133 expression was observed in approximately 20% of well-differentiated Pan-NENs and that higher expression was positively associated with higher TNM stages, more frequent lymph vascular invasion, higher recurrence rates, and shorter disease-free periods. These data suggest that CD133 expression in Pan-NENs is correlated with unfavorable clinical characteristics and could be a potential biomarker to predict tumor progression [77]. Furthermore, Cho et al. reported that in digestive system NENs, 30.3% of well-differentiated NETs, 26.1% of poorly differentiated NECs, and 63.6% of mixed adenoneuroendocrine carcinoma were CD133-positive. However, the study did not find any prognostic significance for CD133 in GEP-NENs [78]. The results from the two studies discussed here imply that CD133 is not abundant in GEP-NENs, but NETs and NECs may share similar expression levels. Clearly, more investigation is needed to evaluate the prognostic significance of CD133 with respect to GEP-NENs.

Activated leukocyte cell adhesion molecule (CD166) is a cell surface protein that plays a role in several adhesive and migratory behaviors, including leukocyte homing, axonal guidance, and cancer metastasis [79]. Tachezy et al. reported that among 38 cases of Pan-NEN samples, 74% of the primary tumors strongly expressed CD166, but only 50% of the metastases exhibited strong CD166 expression. Decreased CD166 expression was significantly correlated with relevant prognostic factors

including tumor size and the presence of distant metastases. On the basis of these clinical findings, CD166 may have biomarker potential for predicting a poor prognosis [80].

Neural cell adhesion molecule (CD56) is a cell surface protein that, as indicated by its name, provides a cell adhesion function. CD56 is expressed in neuroendocrine and neuroectodermal cells as well as their tumors [81]. Importantly, CD56 was identified as a tumor marker for small cell lung cancer (SCLC) and Merkel cell carcinoma, and CD56 serum levels were significantly correlated with the clinical outcomes of SCLC patients [82,83]. Within the context of GEP-NENs, Farinola et al. revealed that an accurate cytopathologic NEN diagnosis could be made by fine-needle aspiration biopsy followed by flow cytometry if CD56 expression occurred concurrently with lymphoid markers during instances of low immunoreactivity [84]. Nonetheless, the role of CD56 in GEP-NENs has not been fully evaluated and warrants further investigation.

3. Comparison of the TIME among different types of GEP-NENs

3.1. Comparison of the TIME among GEP-NENs with different primary sites

According to a review by the Surveillance, Epidemiology, and End Results Program of the National Cancer Institute describing GEP-NEN epidemiology, the rectum is the most common primary tumor site, followed by the small intestine, colon, pancreas, stomach, and appendix [4]. The 5-year survival rates of patients with GEP-NENs varies among the different primary tumor sites. For example, rectal NENs exhibit the highest 5-year survival, but Pan-NENs exhibit the lowest survival rates [4]. These results indicate heterogeneity among primary GEP-NENs, which supports the hypothesis that the TIME of different primary sites also exhibits heterogeneity.

As already discussed, Pan-NENs express higher TILs, PD-1, and PD-L1 than non-pancreatic NENs [27]. In SINENs, duodenal NENs display higher immune infiltration than jejunal or ileal NENs [25]. Higher TIL levels are associated with shorter OS in patients with Pan-NENs and non-pancreatic NENs. Furthermore, increased expression of PD-1 and PD-L1 is significantly associated with shorter OS in non-pancreatic NENs. However, PD-1 and PD-L1 expression is not indicative of overall survival in Pan-NENs [27].

Primary tumors that originate from the stomach, intestinal tract, or pancreas may exhibit significantly different overall patterns of tumor microenvironment and immune infiltration [85]. Understanding the association between different primary tumor sites and TIME characteristics in GEP-NENs may help with prognostic predictions and warrants further investigation.

3.2. Comparison of the TIME between functional and non-functional GEP-NENs

Functional GEP-NENs account for approximately 25–30% of all GEP-NENs, with characteristic clinical symptoms due to hormonal hypersecretion [86]. Early diagnosis and treatment of GEP-NENs can be made when the specific hormonal symptoms are recognized, which can increase survival. The major treatment for functional GEP-NENs is biotherapy including SSAs and in IFNs. Immunotherapy is rarely used for treatment. For this reason, a current goal within the field is to more fully understand the role of the TIME in GEP-NENs to identify novel biomarkers that can be used for immunotherapy. However, studies exploring the TIME composition in the context of immunotherapy in functional GEP-NENs is sparse. Therefore, it is imperative to increase our understanding of the TIME in functional GEP-NENs.

3.3. Comparison of the TIME among G1/G2/G3 GEP-NETs and GEP-NECs

There are distinct differences between GEP-NETs and GEP-NECs with respect to biological behavior, clinical course, and treatment. To treat G1 and G2 NETs, radical surgery and SSA are recommended. In addition, novel targeted drugs including everolimus and sunitinib are commonly used [87,88]. G3 NETs are a new category that present with a well-differentiated morphology and a Ki67 index > 20%. These tumors have limited responsiveness to platinum-based chemotherapy. The major treatment for G3 GEP-NECs is cytotoxic chemotherapy. A deeper understanding of the TIME differences between heterogeneous tumors could be informative for the development of individualized treatments.

Increased TAM infiltration is associated with higher grade GEP-NENs [19]. General T cells, memory T cells, CTLs, and Tregs in G3 GEP-NETs are reported to have similar expression levels in both high- and low-grade tumors [24]. However, a study that enrolled 244 GEP-NEN patients reported that 50% of G3 tumors had high TILs infiltration compared to only 17.1% of G1/G2 tumors that exhibited the same characteristics [27]. Because $CD20^+$ TILs are less prevalent in Pan-NETs than in Pan-NECs, the presence of B cells might contribute to the differences between the two cancers [48]. Pan-NECs have a hot TIME with large numbers of TILs. On the other hand, G1 to G3 Pan-NETs have a cold TIME with little TIL infiltration. Furthermore, with increasing Pan-NEN grade, the number of $PD\text{-}1^{high}$ T cells and $PD\text{-}1^{high}$ M2 TAMs was enhanced, which may be a predictor of poor survival. Of note, the highest number of infiltrating cells in Pan-NECs were $CD3^+CD4^+$ T cells [48]. In summary, GEP-NECs have a hot TIME with greater TIL infiltration than GEP-NETs.

The expression of PD-L1 is strongly associated with the G3 WHO classification and the Ki67 proliferation index. Notably, almost 100% of well-differentiated G3 GEP-NETs express PD-L1 [50]. PD-L2 in G3 GEP-NETs did not significantly differ between either G1 or G2 GEP-NETs [24]. Because of the immunosuppressive characteristic of G3 NENs when compared to lower grade NENs, the application of immune checkpoint inhibitors may be a promising treatment for this subset of patients [48].

3.4. TIME comparison between GEP-NECs and other digestive system cancers

The most distinct characteristic of the pancreatic ductal adenocarcinoma (PDAC) tumor microenvironment is its abundant stroma. In contrast, the Pan-NEN microenvironment is characterized by a high microvessel density. It is well-accepted that an abundant stroma should inhibit immune cell infiltration and that a well-developed vascular supply should contribute to immune cell infiltration. Therefore, it is hypothesized that the TIME of Pan-NECs and PDACs must be distinct.

Takahashi and coworkers quantitatively analyzed TIL numbers and PD-1/PD-L1 expression in Pan-NENs and PDACs with multiplexed fluorescent immunohistochemistry [48]. They observed that Pan-NECs and PDACs had hot TIMEs with large numbers of TILs. Meanwhile, $CD3^+$ and $CD8^+$ T-cell numbers, as well as $CD20^+$ TIL counts were comparable in Pan-NECs and PDACs. Surprisingly, this study suggests that Pan-NECs and PDACs have similar TIMEs.

Because the infiltration of immune cells and molecules was almost identical between Pan-NECs and PDAC, unknown correlations between the two tumor types may exist. Therefore, we speculate that GEP-NECs and digestive system cancers may share infiltration of the microenvironment by immune cells. Applying proven immunotherapeutic treatment options for digestive cancers to GEP-NECs may have profound effects.

4. The interplay between tumor cells and the TIME

Tumor cells can secrete a variety of factors that promote the recruitment of immune cells into the TIME, although to-date, the process has not been investigated in GEP-NENs. The presence of infiltrating immune cells within the GEP-NEN microenvironment, however, does not guarantee a beneficial immune response. It has been reported that

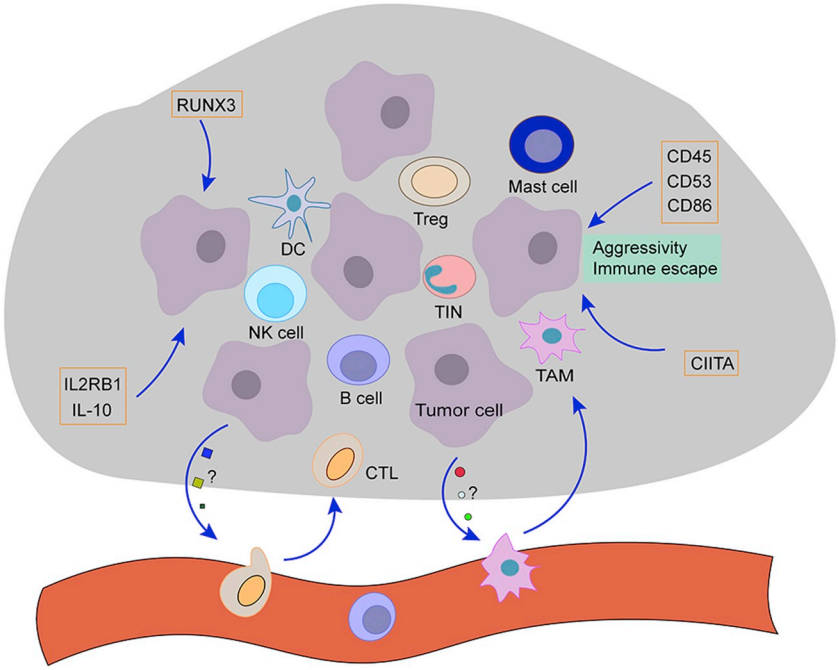

Fig. 2. The interplay between tumor cells and the TIME. Several factors secreted by tumor cells promote the recruitment of immune cells into the TIME. Immunomodulatory factors within the TIME including IL2RB1, IL-10, CD45, CD53, CD86, CIITA, and RUNX3 are master regulators of tumor cell aggressiveness and immune escape. TIME, tumor immune microenvironment; TAM, tumor-associated macrophages; CTL, cytotoxic T lymphocytes; Treg, regulatory T cells; TIN, tumor-infiltrating neutrophils; DC, dendritic cells.

immunomodulatory factors, including IL2RB1, IL-10, CD45, CD53, CD86, CIITA, and RUNX3, are master regulators of tumor aggressiveness and immune escape in neuroendocrine tumors [89]. In conclusion, the TIME of GEP-NENs is hypothesized to be immunosuppressive and suitable for tumor progression; however, the specific effects of tumor cells on the TIME remains poorly understood (Fig. 2).

As in other types of cancers, the complex crosstalk between neoplastic cells and the TIME has been partially elucidated. Alterations of gene expression profiles in tumor cells and tumor-produced cytokines and chemokines could establish an immunosuppressive and pro-tumoral TIME, thus supporting tumor growth and invasion. For example, $BRAF^{V600E}$ in melanoma cells has been shown to decrease production of CCL4 and thus reduce the recruitment of DCs [90]. Additionally, $KRAS^{G12D}$ in PDAC induces expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), which contributes to increase myeloid cells with immunosuppressive functions [91]. Additionally, the TIME also has an important influence on tumor growth, invasion, and metastasis [92]. The overall infiltration and characteristics of T cells within the TIME are the major factors that determine tumor progression, and T-cell exhaustion is reportedly crucial in tumor immunology [93]. Furthermore, TAMs are abundant in most types of cancers and function as pro-tumorigenic and pro-angiogenic players [94,95].

5. Interplay between the tumor immune and stromal microenvironments

A recently published review described a process where extracellular matrix fragmentation in GEP-NENs recruited immune cells due to chemoattractants that were released from matrix remodeling [11]. Bowden et al. [96] conducted a study to determine the role of cancer-associated fibroblasts (CAFs) in the neuroendocrine tumor microenvironment, using three cell culture environments, including the co-culture of stromal cells and CAFs. These investigators reported that CAFs actively secrete monocyte chemoattractant protein 1, IL-6, and VEGF, which contribute to the formation of a tumor-promoting a TIME. Furthermore, TGF-β, which is primarily released by stromal cells, played a dual role in the human NET cell line BON and exhibited both antiproliferative and protumoral effects. These findings indicate that the crosstalk between TIME and the stroma are quite complex, necessitating further studies [57]. The effects of the stromal microenvironment on the TIME are not well characterized, and similarly, the effects of the TIME on stroma modification are also unknown in GEP-NENs. However, there is some evidence suggesting that TINs can increase the bioavailability of VEGF and activate angiogenesis through matrix metalloprotease type 9 secretion (Fig. 3) [97].

Notably, the interplay between stroma and TIME has been reported in other types of cancers. CAFs are one of the most important components in stroma, as these cells secrete a variety of cytokines, chemokines, growth factors, and interleukins and therefore contribute to the immunosuppressive TIME [98]. It has been reported that CAFs express a pro-inflammatory gene signatures and thus contribute to tumor growth by increasing the recruitment of immune cells and promoting neovascularization [99]. Additionally, in a study of melanoma, CAFs suppressed melanoma-specific CTLs via secretion of cyclooxygenase-2 and upregulation of PD-L1 and PD-L2 [100]. Interestingly, it has been reported that the loss of tumor suppressors in stromal cells affect the type and characteristics of immune cells within TIME. Specifically, p53-deficient hepatic stellate cells secrete factors that induce M2 polarization of macrophages, and such polarization is often correlated with immunosuppression [101].

6. Novel treatment options for GEP-NENs

6.1. Targeting the TIME in GEP-NENs

GEP-NENs are a group of slow-growing tumors that are largely asymptomatic. Most patients are diagnosed after their tumor has metastasized, at which point resection is not a viable treatment option. Due to the limited treatment options that are available for GEP-NENs, the development of novel immunotherapeutic approaches is essential. Recently, new therapeutic options that target the TIME have emerged to treat other cancers and may also be applicable to GEP-NENs [53]. Potential targets for GEP-NEN immunotherapy are summarized below (Fig. 1).

PD-L1 was detected in high-grade GEP-NENs and in particular, in GEP-NECs, indicating that patients with these tumors are more likely to benefit from PD-1/PD-L1 immunotherapy [53,102,103]. Based on the characteristics of well-differentiated G3 GEP-NETs, in which PD-L1 expression was almost 100%, PD-1/PD-L1 inhibitors may be a useful alternative therapy for these specific tumors [24]. The KEYNOTE-028

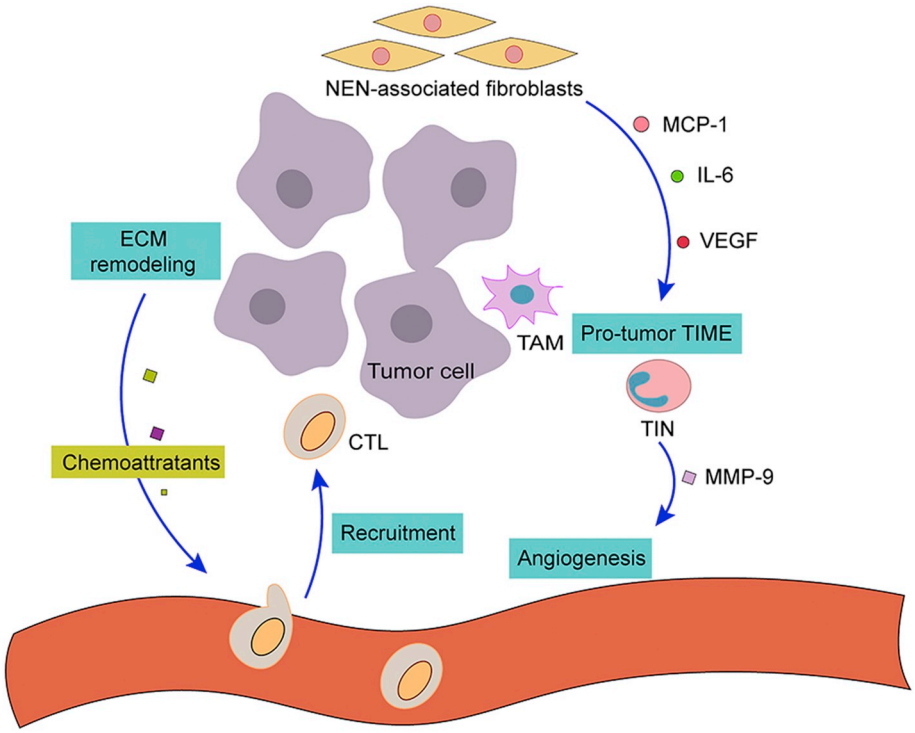

Fig. 3. The interplay between the TIME and the stromal microenvironment. The process of ECM remodeling may recruit immune cells by releasing chemoattractants. NEN-associated fibroblasts in the stromal microenvironment secrete MCP-1, IL-6, and VEGF to contribute to a pro-tumor TIME. TINs within the TIME secrete MMP-9 and activate angiogenesis. ECM, extracellular matrix; MCP-1, monocyte chemoattractant protein 1; VEGF, vascular endothelial growth factor; TIN, tumor-infiltrating neutrophils; TIME, tumor immune microenvironment; MMP-9, matrix metalloprotease type 9.

Table 1
Ongoing clinical trials targeting the PD-1-PD-L1 pathway in GEP-NENs.

Therapeutic intervention	Phase	Patient population	Estimated completion date	NCT number
Avelumab	I/II	GEP-NECs	September 20, 2020	NCT03278405
Avelumab	II	Advanced, G2-3 NETs	September 20, 2021	NCT03278379
Avelumab	II	NECs progressive after chemotherapy	January 2024	NCT03352934
Avelumab	II	Metastatic GEP-NECs	February 2020	NCT03147404
Durvalumab +Tremelimumab	II	Advanced GEP-NENs or lung origin	April 2020	NCT03095274
Ipilimumab + Nivolumab	II	Advanced, progressive, well-differentiated non-functional GEP-NETs or NETs of lung	January 15, 2024	NCT03420521
JS001	Ib	Advanced NETs with Ki-67 ≥ 10%	June 2019	NCT03167853
PDR001	II	Advanced or metastatic, well-differentiated, non-functional GEP-NETs, or thoracic origin or GEP-NECs	April 3, 2020	NCT02955069
Pembrolizumab+ Somatuline Depot	Ib/II	Non-resectable, recurrent, or metastatic well- or moderately differentiated GEP-NETs	June 1, 2020	NCT03043664
Pembrolizumab	II	Metastatic, high-grade NETs with Ki67 > 20%	January 2020	NCT02939651
Pembrolizumab	II	Metastatic or unresectable NECs of non-pulmonary origin, high-grade with Ki-67 > 20%	September 2022	NCT03190213
Pembrolizumab	II	Advanced NECs or G3 NETs	December 2021	NCT03290079

study, which enrolled patients with advanced carcinoid and Pan-NENs, showed an objective response rate in 12% of patients with carcinoid and 6% of patients with Pan-NEN following treatment with pembrolizumab (10 mg/kg for 2 weeks). Additionally, stable disease was reached in 60 and 88% of carcinoid and Pan-NEN patients, respectively [53]. Sparalizumab (PDR-001) is a PD-1 inhibitor that was recently reported to have similar outcomes in GEP-NEN patients, excluding those with G3 GEP-NETs [11]. While these therapeutics are promising, 12 additional clinical trials exploring immunotherapeutic targeting of the PD-1-PD-L1 pathway in GEP-NENs are currently underway (Table 1).

TAMs promote an immunosuppressive tumor microenvironment and are a potential target for blockade therapy [104]. Krug and colleagues used the neuroendocrine mouse model, RIP1Tag2, to target TAMs with liposomal clodronate, which disrupted tumor progression and significantly reduced tumor angiogenesis [105]. Also within the context of the RIP1Tag2 mouse model, Rebastinib, which is a selective inhibitor of the angiopoietin receptor Tie2, reduced $Tie2^{+}$ macrophage counts, microvessel density, and liver metastases, all within the TIME [106]. A study by Antonios and colleagues reported that inhibiting PD-1 and the colony-stimulating factor 1 receptor decreased TAM immunomodulatory functions. Subsequently, the antitumor immune response of T cells was activated and contributed to a tumoricidal outcome [107]. These finding suggest that targeting TAMs may be a novel therapeutic approach in highly angiogenic tumors, such as GEP-NENs. Additionally, the association between TAMs and PD-1/PD-L1 presents a novel treatment option in immune-oncology. A combined approach that includes PD-1 monoclonal antibodies and a colony-stimulating factor 1 receptor inhibitor could be a promising therapeutic approach for GEP-NENs.

The role of Tregs in immune suppression and as predictors of poor survival has sparked interest in eliminating or targeting Tregs to overcome the immunosuppressive tumor microenvironment. To this end, CD25 is suggested as a promising target for Treg depletion [108]. Tregs are closely associated with PD-L1, which is generally regarded as critical to their development and maintenance. High PD-L1 expression and increased Treg infiltration are associated with increased histological grade, and tumors with simultaneous high-level expression levels

of both markers have the worst breast cancer prognosis [109]. A study by Dyck and colleagues revealed that high PD-L1 expression was associated with increased Treg infiltration and decreased CTLs. In the same study, anti-PD-1 immunotherapy reduced Treg levels and enhanced the tumoricidal ability of CTLs [110]. Recently, it was also reported that anti-CD25 combined with anti-PD-1 immunotherapy eradicated tumors [108]. To summarize, a combination of Tregs and PD-L1 targeting is a promising approach to treat GEP-NENs, but more research is needed to evaluate the clinical efficacy of these treatments.

NK cell-based immunotherapy to treat cancer has recently gained interest. For example, scientists have initiated clinical trials where haploidentical NK cells are infused into patients with acute leukemia and solid tumors (NCT00582816). Ex vivo-expanded NK cells are also being explored as a treatment option for patients with non-small cell lung cancer (NSCLC) (NCT02118415). A study investigating the combinatory effects of irreversible electroporation and NK cell infusion for advanced pancreatic cancer is also underway (NCT02718859). Despite the promise of these clinical trials, tumor cells are able to escape NK cell-mediated immune surveillance and the specific role of NK cells in GEP-NENs has not been identified. NK cell-based adaptive immunotherapy for the treatment of GEP-NENs remains largely unexplored; therefore, further research in the area is warranted.

Higher CD73 expression is associated with increased GI-NEN malignant potential. Intriguingly, CD73 expression status is also closely related to PD-L1 expression [49]. Furthermore, CD73 is a predictive biomarker for anti-PD-1 immunotherapy [111], and targeting CD73 enhances the therapeutic effect of anti-PD-1 and anti-CTLA-4 monoclonal antibodies [112]. To summarize, CD73 is an important immune molecule and is closely correlated with PD-L1. These data indicate that CD73 could be a novel therapeutic target for GEP-NEN treatment and a potential predictive biomarker for anti-PD-1 immunotherapy.

Four clinical trials (NCT01237678, NCT02420873, NCT00991562, NCT02452554) assessing the safety and efficacy of IMGN901, a CD56-targeting antibody-drug conjugate had controversial results. A combination of IMGN901 and carboplatin/etoposide did not demonstrate improved efficacy in SCLC patients. In contrast, results with the single agent, IMGN901, in Merkel cell carcinoma were encouraging [113,114]. The CD56-targeting antibody-drug requires further investigation to evaluate its effect on GEP-NENs.

CXCR4 and VEGF-A could also be potential therapeutic targets for GEP-NENs and were already discussed in the section describing the cytokine and chemokine milieu.

6.2. Vaccines

Vaccines containing tumor antigen-loaded DCs are a newly emerging and potent form of cancer immunotherapy [115]. A recent clinical study showed that DC vaccine-based immunotherapy combined with chemotherapy was partially effective in patients with advanced PDAC [116]. Schott et al. reported a promising outcome in a patient with Pan-NEC. More specifically, tumor lysate-pulsed DCs initiated a strong T-cell immune response and tumor regression, with a robust perivascular and epidermal infiltration of CD4 and CD8 positive cells. This encouraging case study indicates great therapeutic potential for DC vaccines [117]. However, these results are not validated by clinical trials. The major limitations of DC vaccines include the absence of defined tumor antigens and a lack of standard immunization techniques to induce an antitumor response. Further research is needed to explore the treatment potential of DC vaccines for GEP-NENs, both alone and in combination with other more established treatments.

6.3. Potential biomarkers for the immune response

A crucial variable in immunotherapy efficacy is patient selection. Namely, patients who are sensitive to the treatment should be selected, which requires appropriate biomarkers. The most well-studied biomarkers for PD-1/PD-L1 immunotherapy are PD-L1 expression, mismatch repair deficiency, the tumor mutation load, and lymphocyte infiltration. Unfortunately, only a few of these biomarkers have established roles in GEP-NENs.

Immune checkpoint inhibitors may not be a favorable treatment option for low-grade GEP-NENs due to low PD-L1 expression. In contrast, patients with GEP-NECs are more suitable for immunotherapy due to their large tumor mutation load and hot TIME that contains elevated TIL numbers [118].

The number and antitumor activity of $CD8^+$ CTLs increases after immune checkpoint therapy [119]. A high tumor mutation burden results in increased neo-antigens that drive tumor infiltration by immune cells; therefore, immunotherapy might augment this effect. Kamphorst and colleagues reported proliferation of PD-1^+ $CD8^+$ T cells in the peripheral blood of up to 70% of patients with NSCLC after PD-1 blockade therapy. And better clinical outcomes were observed in patients that exhibited enhanced PD-1^+ $CD8^+$ T-cell proliferation within 4 weeks after PD-1 immunotherapy [120]. Therefore, it was proposed that $CD8^+$ CTLs in the peripheral blood could be used to predict the T-cell response that is induced by PD-1 blockade therapy. The correlation between PD-1 immunotherapy and CTLs in GEP-NENs warrants further research to identify the predictive significance of $CD8^+$ CTLs. These data would contribute to the appropriate selection of patients that are sensitive to PD-1/PD-L1 immunotherapy.

7. Conclusions and perspectives

GEP-NENs are rare, poorly understood tumors that are increasing in prevalence. Although our understanding of the biology and treatment landscape of GEP-NENs is growing, the role of the TIME remains unclear. Therefore, a comprehensive analysis of the TIME is necessary. In this review, we highlighted new insights related to the role of infiltrating immune cells, immune checkpoints, the cytokine and chemokine milieu, and the immunomodulatory factors of GEP-NENs. Our analysis described the heterogenous TIME that exists among different types of GEP-NENs, discussed the interplay among the TIME, tumor cells and the stromal environment, and summarized the feasibility of novel treatment options with respect to the TIME.

Although emerging evidence suggests an important role for the TIME in promoting cancer progression, a comprehensive understanding of the TIME in GEP-NENs is still missing. We described an intriguing phenomenon where some metastases became enlarged and others were reduced after the same treatment, even in the same patient. These data underscore tumor heterogeneity, and the differences among the TIME in metastases may explain the different therapeutic responses. A different TIME between primary tumor and metastatic sites is hypothesized to contribute to different responses to the same treatment. How the TIME evolves during the process of tumor progression and metastases is of great importance and remains unknown. In addition, a clear understanding of TIME remodeling after treatment can aid with the optimization of precision medicine. Continuing to evaluate the TIME of GEP-NENs, not only in experimental research, but also in clinical practice will increase our understanding of its importance. Moreover, a comprehensive analysis of the GEP-NEN TIME can provide crucial information to inform therapeutic development.

The whole-genome landscape of Pan-NENs has been described previously [9], which suggests that the TIME landscape could be characterized by next generation sequencing to elucidate relevant molecular components and to inform GEP-NEN treatment. Currently, the TIME of GEP-NENs is sparsely described in the literature. However, new insights are anticipated to yield important developments for the clinical management of GEP-NENs.

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Declaration of Competing Interest

None.

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