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Review

The World of Immunotherapy Needs More Than PD-1/PD-L1—Two of the New Kids on the Block: LAG-3 and TIGIT

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Simple Summary: In the last decade, immunotherapy has advanced the treatment of solid tumors. Immune checkpoint inhibitors like anti-PD-1/PD-L1 have shown promising results, but many patients face side effects and may develop resistance. This has led to the search for new immunotherapy targets that are effective and safe. Two promising targets are LAG-3 and TIGIT, which interact with the immune system. These can be targeted with drugs, either alone or in combination with anti-PD-1/PD-L1, showing safe profiles. This review explores the immune mechanisms of LAG-3 and TIGIT, their detection through immunohistochemistry, and their potential use in clinical treatments.

Abstract: Immunotherapy has paved the way for the development of solid tumor new treatments in the last decade. The approval of immune checkpoint inhibitors such as anti PD-1/PD-L1 provided a revolution with optimal results. However, a considerable proportion of patients experience adverse therapeutic effects, and up to 50% may develop secondary resistance in the first three to five years. This has prompted the need for identifying new targets for immunotherapy that have good tolerance and biosafety and, of course, good tumoral response, either alone or in combination. Two of these new targets are the Lymphocyte-activation gene 3 (LAG-3) and the T cell immunoglobulin and ITIM domain (TIGIT). They are responsible for several interactions with the immune system, prompting an immunosuppressive phenotype in the tumor microenvironment. Both LAG-3 and TIGIT can be druggable, alone or in combination with anti-PD-1/PD-L1, with rather safe profiles making them attractive. In this review, we highlight some of the immune mechanisms of TIGIT and LAG-3 and their detection by immunohistochemistry, providing some insight into their use in the clinical setting.

Keywords: immunotherapy; PD-1/PD-L1; TIGIT; LAG-3

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1. Introduction

The discovery and introduction of immune checkpoint inhibitors created a new standard in cancer treatment. The tumor microenvironment (TME) is composed of multiple cells, including fibroblasts, endothelial cells, and several types of inflammatory cells [1]. A comprehensive and deeper knowledge of the TME, a feature unique to each tumor, has implications for tumor initiation, progression, metastases, and response to therapy [2].

The approval in 2011 by the Food and Drug Administration (FDA) of the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-ligand 1 (PD-L1), and programmed cell death protein (PD-1) for the treatment of solid tumors [3–6] introduced a new class of drugs, either alone or in combination, into clinical practice, with very good results [7–16]. However, there have been reports of toxicities [17–19] and drug

resistance [20–22], and thus new agents have been brought into consideration [23–27], providing new tools in the clinical setting.

Two of the new agents are the lymphocyte-activation gene 3 (LAG-3) and the T cell immunoglobulin and ITIM domain (TIGIT). In this manuscript, we describe their mechanism of action and their detection by immunohistochemistry.

2. Lymphocyte-Activation Gene 3 (LAG-3)

LAG-3 is a transmembrane protein type I and a member of the immunoglobulin superfamily with an extracellular region composed of four domains, sharing 20% amino acid sequence homology with CD4 [28,29]. LAG-3 was first discovered in 1990 by Triebel and colleagues, it is encoded by a gene in chromosome 12 adjacent to the CD4 gene, explaining their share of homology [30]. This similarity is restricted to the extracellular component [31].

LAG-3, also known as CD223, binds to major histocompatibility complex II, but with a stronger affinity than CD4 [32]. LAG-3 is a negative regulator of immune response, and it is associated with the suppression of T cell activation and cytokine secretion, inducing immune homeostasis; thus, it has the potential to be a promising immune checkpoint (Figure 1) [33].

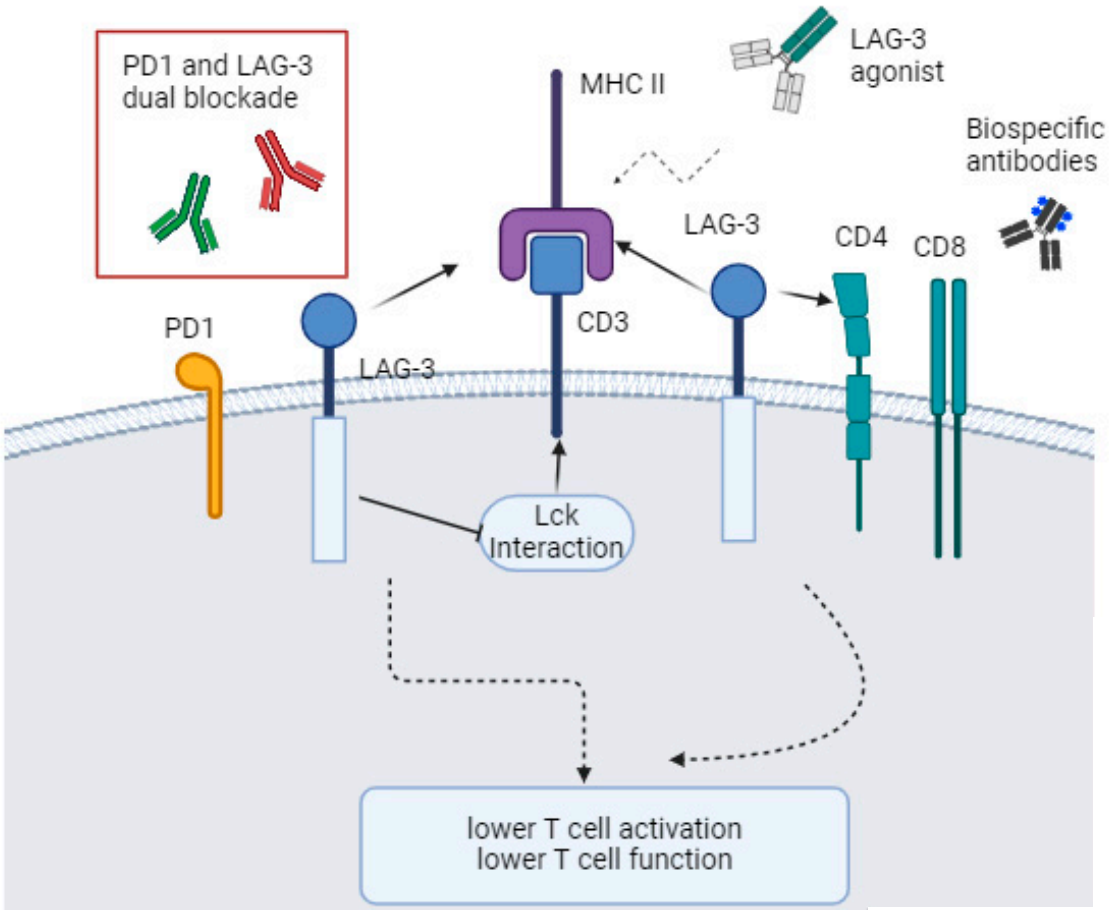

Figure 1. Simple schematic representation of LAG-3 and its interactions. LAG-3 can interact with MHC class II and CD4, among others. This interaction leads to T cell inhibition. Several options have been developed for LAG-3 targeted therapy, such as using LAG-3 agonists and combined treatment with anti-PD-1-specific antibodies. Figure adapted from Andrews LP et al. (https://doi.org/10.1158/1078-0432.CCR-21-2390, accessed on 20 May 2024). Created using BioRender.com, accessed on 20 May 2024.

In the TME, the intra-tumoral lymphocytes (TILs) are an essential component and are composed of CD4+ T cells, CD8+ T cells, regulatory T cells (Tregs), and NK cells, among others. Over-expression of LAG-3 has been reported in TILs, supporting its immune regulatory function [32]. The up-regulation of LAG-3 is usually associated with features of chronic immune exhaustion of CD4+ T cells [34], and the same phenomenon is observed in CD8+ T cells, with an impact that is independent of CD4+ cells [35–37]. The CD8+ T cells' interaction with LAG-3 is complex, and recently, there has been evidence that there are LAG-3 ligands, galectin-3, and liver sinusoidal endothelial cell lectin that may abolish the antitumoral activity of the CD8+ T cells [38–40], without the intervention of the major histocompatibility complex II.

The existence of several inhibitors of immune checkpoints in concurrence with LAG-3 can also negatively affect the LAG-3 antitumoral response [41].

Regarding NK cells, a higher interference of LAG-3 is described in cells that express both NK and T markers, so-called NKT cells. LAG-3 is associated with an exhaustion of these cells in the TME [42].

LAG-3 also has effects in Tregs. Tregs are responsible for disrupting the antitumoral response, mainly by impairing cytokine production [43]. LAG-3 expression is associated with Tregs' differentiation, and LAG-3 blockage is related to lower Tregs' induction [44,45]. Tregs are also associated with FoxP3 expression [46], so LAG-3 therapy may be an excellent therapeutic tool in patients whose tumors present higher expression of these cells in their TME.

LAG-3 expression has been demonstrated in several types of cancer, namely colorectal cancer [47], gastric cancer [48], breast cancer [49], and even pancreatic cancer [50], and it is closely related to their prognosis [51]. Thus, blocking LAG-3 and inhibiting its antitumoral activity may help to regain the function of T cells, namely their cytotoxic activity, and reduce the Tregs activity in suppressing the immune response; consequently, tumor destruction may be induced [52].

Interestingly, LAG-3 has a strong synergy with PD-1, with reports of a strong antitumoral response in patients with melanoma who are resistant to anti-PD-1 alone [53]. This dual blockage has combined antitumoral effects, such as inhibition of Tregs activity, promotion of maturation of dendritic cells, and salvaging dysfunctional CD4+/CD8+ T cells [54].

Currently, more than 80 clinical trials are evaluating several drugs targeting LAG-3, including monoclonal antibodies, double antibodies, and small molecules directly targeting LAG-3 molecules or their ligands [55,56]. Some trials are testing LAG-3 in combination with anti-PD-1/PD-L1 molecules showing promising results [57],. LAG-3 is sometimes referred to as the third checkpoint inhibitor [58].

A recent and very interesting clinical trial—RELATIVITY-047 [56,59]—compared the combination of nivolumab and relatlimab with nivolumab alone in patients diagnosed with inoperable or metastatic melanoma. The study showed a longer progression-free survival in patients (median follow-up of 13.2 months) with double therapy (10.1 months) when compared with patients with only nivolumab (4.6 months)

This led to the approval by the FDA in 2022 of the use of a combination of two immunotherapy drugs (Opdualag) for the treatment of patients with advanced melanoma: relatlimab and nivolumab. This is a hallmark because relatlimab is the first FDA-approved drug to block the activity of LAG-3.

This dual therapy is under ongoing investigation for gastroesophageal cancer in a phase Ib trial (NCT03044613) [60] in patients with resectable tumors. The OS at 2 years in the combination arm was superior (82.6% vs. 72.5%), and LAG-3 expression was associated with better pathological response.

In advanced gastric cancer, a phase Ib trial (NCT02720068) [60] reported very exciting yet preliminary results in patients with PD-1-naïve gastric cancer. Favezelimab (an anti-LAG-3 drug) had a safe profile and an interesting objective response rate, especially in patients with PDL1 CPS $\geq$ 1 tumors and with a higher dose. This clinical trial is also

investigating the effects of the combination therapy in other solid tumors such as head and neck squamous cell carcinomas.

In colon cancer, combined therapy is already ongoing. Garralda et al. [61], showed that the combination therapy with favezelimab and pembrolizumab had a manageable safety profile, with promising antitumoral activity in patients with microsatellite stable and PD-L1 CPS $\geq$ 1 tumors. Still in colon cancer, a phase 2 clinical trial iSCORE (NCT03867799) [62] is investigating the antitumoral benefit of nivolumab (anti-PD1) and relatlimab (anti-LAG3) therapy in patients with metastatic CRC, RAS/BRAF wildtype and without microsatellite instability. Clinical data are still scarce, but there seems to be benefit in disease control.

The antitumoral effect of anti-LAG-3 has also been evaluated in a pan-solid tumor context. One phase I/II study NCT02460224 [63] has assessed the safety and antitumoral effects of the LAG-3 inhibitor, ieramilimab (LAG525), with the anti-programmed cell death-1 antibody, spartalizumab, in advanced/metastatic solid tumors. Once more, the combination therapy showed a safe profile and good antitumoral response. A list of all the clinical trials using LAG-3 reported in the text can be found in Table 1.

Table 1. List of clinical trials described in the article using LAG-3.

Clinical Trial	Status	Summary	Reference
KEYFORM-008	Ongoing	Favezelimab/pembrolizumab with bendamustine or gemcitabine in participants with PDL1-refractory, relapsed, or refractory classical Hodgkin lymphoma.	[57]
RELATIVITY-047 (NCT03470922)	Ongoing	Relatlimab in combination with nivolumab in treating unresectable melanoma or melanoma that has spread.	[56,59]
NCT03044613	Ongoing	Nivolumab (anti-PD-1) or Anti-PD1/Anti LAG-3- (relatlimab) as a neoadjuvant in patients with resectable distal esophageal/gastroesophageal junction cancer.	[60]
iSCORE (NCT03867799)	Ongoing	Antitumoral benefit of nivolumab (anti-PD1) and relatlimab (anti-LAG3) therapy in patients with metastatic colorectal, RAS/BRAF wildtype and without microsatellite instability	[62]
NCT02460224	Completed	Assessment of safety and antitumoral effects of the LAG-3 inhibitor, ieramilimab with the anti-PD-1 antibody, spartalizumab, in advanced/metastatic solid tumors.	[63]

3. T Cell Immunoglobulin and ITIM Domain (TIGIT)

T cell immunoglobulin and ITIM domain (TIGIT) was discovered in 2008, and it is regulated by the *TIGIT* gene located at chromosome 3. TIGIT is a transmembrane protein, type 1 poliovirus receptor (PVR), and part of the nectin and nectin-like receptors superfamily, with a single extracellular domain and a single ITIM domain [64]. TIGIT interacts with four ligands: nectin and nectin-like adhesion molecules CD115, CD112, CD113, and nectin-4 (Figure 2) [65].

TIGIT expression is found mostly in CD4+CD25^{hi} Treg cells, activated T cells, NK and NKT cells, and memory T cells; naïve CD4+ T cells do not exhibit TIGIT expression, but its expression can be induced after activation [66]. The difference in the inflammatory cells may explain the different response to TIGIT antibodies in solid tumors.

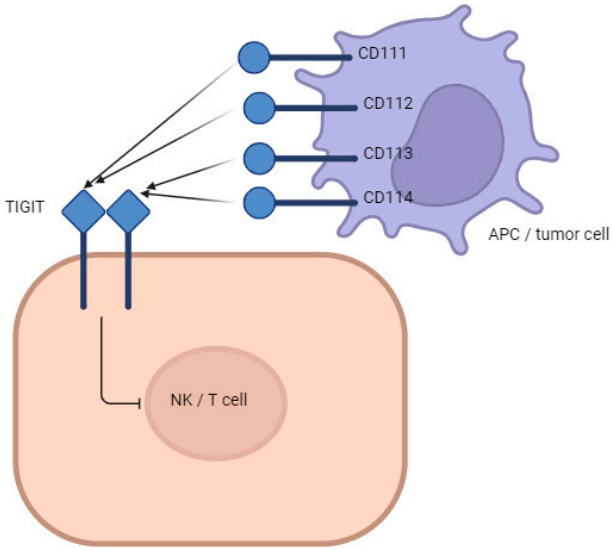

Figure 2. Simple representation of TIGIT interactions. Through several clusters of differentiation, the antigen presenting cells or tumor cells interact with TIGIT, and this activation inhibits the NK or T cell functions. Direct blockade of these interactions is, therefore, a promising therapeutical target. Adapted from Yue C et al. (https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2022.911919/full, accessed on 20 May 2024). Created with BioRender.com, accessed on 20 May 2024.

Due to TIGIT's expression profile in NK cells, Tregs, and cytotoxic T cells, it is considered an important target. TIGIT is a negative regulator of immune response, with higher affinity to CD155 than its costimulatory receptors, CD226 and CD96 (expressed in T cells), outcompeting them [67]. TIGIT connection enables the survival of T cells, and when activated in NK cells, it inhibits their cytotoxic activity by diminishing IFN-γ production and liberation [68,69]. TIGIT is associated with a perturbation of the cytokine balance, prompting a Th2 phenotype, but it is not associated with an autoimmune response when knocked out, like CTLA-4 and PD-1 [70]. In NK cells, there is a preferential expression of TIGIT in CD16+ NK cells, contrasting with other PVR-like receptors [71]. Therefore, TIGIT influences the survival and exhaustion of NK cells and is associated with the mediation of T cell depletion [72].

Regarding T cells, an elevated expression of intratumoral TIGIT CD8+ cells has been pointed out as a predictive biomarker of clinical outcome, being associated with good response to adjuvant chemotherapy in bladder cancer, according to the study of Liu Z et al. [73]. On dendritic cells, the TIGIT induced these cells to express an immature immunogenic phenotype by CD155 activation, thus impairing the activity of dendritic cells in antigen presentation and T cell activation [66].

In Tregs, the expression of TIGIT is constitutively expressed [74] and associated with a consistent FoxP3 activity, FOXO1 nuclear expression, and a higher suppression of function, contributing to a cold TME [75].

Anti-TIGIT therapies have been a promising target in the clinical setting in several tumors, such as gastrointestinal tract tumors, melanoma, biliary tract cancer, colorectal cancer, urothelial cancer, and liver cancer, among others, with several phase II and phase III clinical trials currently ongoing [76–80]. TIGIT blockade reinvigorates the anti-tumor T cell responses, enhances anti-tumoral NK effects, and reduces the suppressive effect of the tumor-infiltrating Tregs [81].

Like in LAG-3, the TIGIT blockade has a synergistic effect with anti-PD-1/PD-L1 blockers and provides a major increase in the anti-tumoral CD8+ T cells' action [82]. The anti-PD-1/PD-L1 mechanisms of resistance are rather well described and include several possibilities, from immune to non-immune; the upregulation of immune coinhibitory

molecules, such as LAG-3, VISTA, TIM-3 or TIGIT, account for a significant number of these mechanisms, and therefore it should be no surprise that the co-inhibition provides interesting results [83]. TIGIT is the most frequently co-expressed immune checkpoint receptor in PD-1+ CD8+ T cells [84]. The PD-1 blockade in some cases is expected to increase the expression of TIGIT by 1.5 times, making it an interesting target [85]. Hence, it is no surprise that several clinical trials are exploring this dual blockade [82,86–89].

Animal models are exploring these possibilities [90]. Monteran L. et al. [91] showed that combining TIGIT blockade with myeloid-derived suppressor cells was able to activate antitumoral immunity and attenuate bone metastases' formation. The findings from Han JH et al. [92] reinforced this. In their paper, the authors state that anti-TIGIT antitumoral effects are not attained only by depletion of intratumoral Tregs (or any other population TIGIT+), but they are mediated by activating signals from the myeloid cells.

Anti-TIGIT has also been described as having a synergistic effect with radiotherapy [93]—the authors of this study reported the synergistic effects of RT combined with anti-TIGIT therapy in an animal model and in a cohort of patients with esophageal squamous cell carcinoma. Interestingly, an upregulation of TIGIT following RT was found, and the administration of the anti-TIGIT antibody increased RT efficiency. Through knockout gene techniques they also demonstrated that CD103+ dendritic cells were necessary to promote the anti-tumoral effects of the combination scheme, thus prompting additional immune environment modulation in order to maximize therapy effects.

The phase 1a/1b GO30103 open-label study [94] was able to demonstrate a promising antitumoral activity of the combination tiragolumab plus atezolizumab in patients with immunotherapy-naive non—small cell lung cancer and esophageal cancer. This was also demonstrated by the phase III study SKYSCRAPER-08 [95], in which the combination of tiragolumab plus atezolizumab plus chemotherapy demonstrated statistical and clinical improvement in OS (15.7 vs. 11.1 months) and in PFS (6.2 vs. 5.4 months), even taking into consideration PD-L1 status.

The combination of tiragolumab plus atezolizumab has also been under trial in "Previously Untreated Locally Advanced Unresectable or Metastatic PD-L1-Selected Non-Small Cell Lung Cancer" (study SKYSCRAPER-01, NCT04294810), and early results demonstrate a clinical benefit, but without statistical significance [96]. The use of anti-TIGIT in lung cancers had been previously addressed by Niu J et al. [79]. This study evaluated the efficacy of the anti-TIGIT antibody vibostolimab as monotherapy or in combination with pembrolizumab in patients with advanced solid tumors, including non-small cell lung cancer (NSCLC). The combination therapy showed promising results with better response rates than monotherapy, including NSCLC, independent of PD-L1 status.

A pan-cancer study is ongoing in Japan—jRCT2080224926 [97]. This study is still in its beginning stages but it is assessing tiragolumab in combination with atezolizumab in Japanese patients with advanced or metastatic solid tumors. So far, only three patients with non-small cell lung cancer, pancreatic cancer, and cholangiocarcinoma have been assessed.

In colorectal cancer, combination therapy has also been studied. An ex vivo study assessed the capacity of the combination of atezolizumab (anti-PD-L1) and tiragolumab (anti-TIGIT) to restore immune response of TILs in microsatellite stable CRC [98]. When used in combination, there was a reactivation of CD8+ T cells in 46% of the microsatellite stable CRC. This was associated with high expression of CD96 on T cells, which could work as a surrogate marker for therapy efficiency. The dysfunction of T cells due to TIGIT had already been reported by Shao Q et al. [99], more specifically in CD3+ T cells, which could be restored with an anti-TIGIT antibody and boosted by combination with anti-PD-1. A list of all the clinical trials using TIGIT reported in the text can be found in Table 2.

Table 2. List of clinical trials described in the article using TIGIT.

Clinical Trial	Status	Summary	Reference
CITYSCAPE (NCT03563716)	Ongoing	Efficacy study of tiragolumab plus atezolizumab compared with atezolizumab in chemotherapy-naive patients with locally advanced unresectable or metastatic PD-L1-selected non-small cell lung cancer.	[76]
SKYSCRAPER-01 (NCT04294810)	Ongoing	Compared the efficacy and safety of tiragolumab plus atezolizumab compared with atezolizumab alone as a first line treatment with PD-L1 high locally advanced, unresectable or metastatic non-small cell lung cancer.	[96]
SKYSCRAPER-02 (NCT04256421)	Ongoing	Evaluated the efficacy of tiragolumab plus atezolizumab and carboplatin and etoposide compared with atezolizumab and etoposide in participants with chemotherapy-naive extensive-stage small cell lung cancer.	[77,78]
KEYVIBE-001 (NCT02964013)	Ongoing	Safety, efficacy, and pharmacokinetics study of vibostolimab isolated or in combination with pembrolizumab or pembrolizumab plus pemetrexed and carboplatin in metastatic solid tumors for which there is no therapy	[79]
Morpheus-Liver (NCT04524871)	Ongoing	Study on locally advanced or metastatic hepatocellular carcinoma patients who have not received prior systemic therapy for their disease.	[80]
EDGE-Gastric (NCT05329766)	Ongoing	Treatment combinations with and without chemotherapy in participants with locally advanced unresectable or metastatic gastric, GEJ, and esophageal adenocarcinoma. Chemotherapy will consist of FOLFOX (oxaliplatin, leucovorin, fluorouracil).	[86]
ARTEMIDE-01 (NCT04995523)	Ongoing	Phase I/II study designed to evaluate if experimental anti-TIGIT/anti-PD-1 bispecific antibody rilvegostomig is safe, tolerable and efficacious in participants with advanced or metastatic non-small cell lung cancer.	[89]
SKYSCRAPER-08	Ongoing	A phase III study of atezolizumab plus tiragolumab in combination with paclitaxel and cisplatin compared with paclitaxel and cisplatin as a first-line treatment in patients with unresectable locally advanced, unresectable recurrent, or metastatic esophageal squamous cell carcinoma.	[95]
GO30103 (NCT02794571)	Ongoing	Evaluated the safety, tolerability of tiragolumab alone or in combination with atezolizumab and/or other therapies in locally advanced, recurrent, or metastatic incurable tumors.	[94]
jRCT2080224926	Completed	Tiragolumab and atezolizumab intravenously every 21 days until unacceptable toxicity or disease progression.	[97]

4. Triple Blockade on the Way?

We have stated the advantages of dual inhibition in immunotherapy, but also referred that there are several immune checkpoint receptors interacting at the same time in the inflammatory cells, which can be modified by the inhibition of their neighbors [100]. Therefore, one possible answer is blocking several of them simultaneously [101]. A very recent and interesting work from Yang R et al. [102] is in line with this rationale. The authors developed a tri-specific antibody targeting PD-L1, TIGIT and LAG-3, which promoted greater T cell expansion and anti-tumoral activity when compared to benchmark data.

5. How to Assess LAG-3 and TIGIT Expression?

So far, no consensus has been reached as to when LAG-3 and TIGIT should be tested by immunohistochemistry, with many trials selecting patients with tumor progression after anti-PD-1/PD-L1 blockade. Since this is a non-personalized approach, the immunohistochemical detection of these markers may be the most accurate method since it can assess their expression and also their spatial distribution.

Immune checkpoint markers are difficult for pathologists to evaluate. A standardized method of evaluation should be introduced in order to know which of the scores is the most useful—the tumor positive score (TPS) vs. the combined positive score (CPS) [103,104]. Alternatively, should only the intensity score be used? Should the intensity multiplied by the distribution of cells, independent of staining intensity be used? [105] There are several reported scores in the literature regarding immune checkpoint evaluations, and without a consensus statement, evaluations will be poorly reproducible [106,107]. The Society for Immunotherapy of Cancer (SITC) has published guidelines [108], and in the future, a strict methodology should be available, especially when introducing multiplex technology associated with imaging analysis and quantification tools [109].

Nevertheless, some antibodies have been developed and have a good reproducibility for LAG-3 in melanoma [110,111], mesothelioma [112] and gastric cancer [113]. Immunohistochemical expression of TIGIT has been described in Hodgkin's lymphoma [114,115] and solid tumors [116,117], with some cases evaluating it on tumor cells and others in peritumoral inflammatory cells. An example of immunohistochemical staining can be seen in Figures 3 and 4.

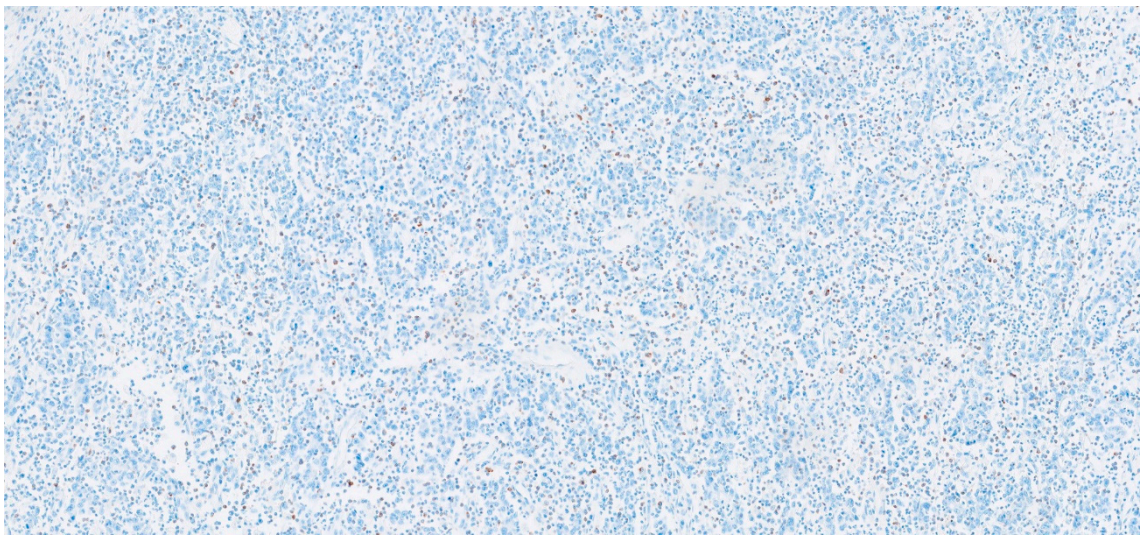

Figure 3. LAG-3 immunohistochemical staining in a medullary gastric cancer. There are several scattered inflammatory cells with positive staining. Medullary cancers are rather special since they are usually associated with microsatellite instability and PD-L1 expression; thus, they may be candidates for double therapy.

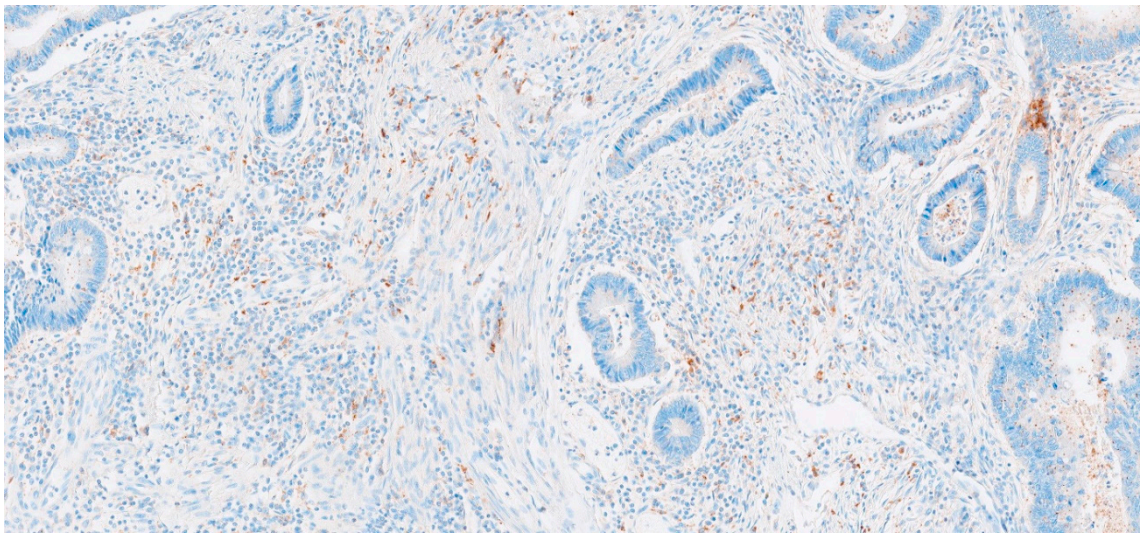

Figure 4. TIGIT immunohistochemical staining in a colorectal cancer. There is visible staining in the inflammatory cells associated with the tumor.

Recently, some studies have assessed the immunohistochemical expression of these markers and their clinical and pathological correlation. A study from 2023, from Tavana S et al. [118] presented some interesting findings. In 136 patients with colorectal cancer, they described that LAG3+ TILs are less frequent than CD3+ and CD45RO+, but there was a correlation between a higher number of LAG3+ TILs in the invasive margin and a higher T stage.

6. New Strategies

Despite advances in immunotherapy, there are still many hurdles to overcome, namely toxicity and the resistance to immunotherapy blockade, either intrinsic or acquired. It has been shown that an abnormal glucose metabolism of tumors has an important role [119], and an acidic environment promotes tumor growth and metastases as well as therapy resistance due to the blocking of T cell activation; thus, immune cells do not mount an effective response under these conditions [120–123]. A recent strategy is the neutralization of tumor acidity of the microenvironment with an increased response and efficacy of immunotherapy due to an increase in CD8+ and CD4+ T cell infiltration in tumor tissues [120].

7. Conclusions

Therapy with immune checkpoint inhibitors is evolving at a rapid pace, with an urgent need for new markers. The incorporation of druggable targets like LAG-3 and TIGIT for therapy, alone or combined, should be a fast-approaching clinical reality. Oncology-related health professionals should be aware of these possibilities, especially pathologists, who soon may be asked to evaluate these markers for therapeutic purposes.

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