



The Paradox of Cancer Immune Exclusion: Immune Oncology Next Frontier

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Abbreviations

ACT	Adoptive Cell Therapy
CAF	Cancer-Associated Fibroblast
CIT	Checkpoint Inhibitor Therapy
CSC	Cancer Stem Cells

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DST	Dystonin
GC	Germinal Center
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
ICD	Immunogenic Cell Death
ICR	Immunologic Constant of Rejection
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IO	Immune Oncology
PD1	Programmed cell Death protein-1
PDL1	Programmed Death Ligand-1
PI3K	Phosphatidyl-Inositol-4,5-bisphosphate 3-Kkinase
SIRP-α	Signal Regulatory Protein- α
STAT	Signal Transducer and Activator of Transcription
TAMs	Tumor-Associated Macrophages
TGF	Transforming Growth Factor
TIL	Tumor Infiltrating Lymphocytes
TIS	Tumor Inflammation Signature
TME	Tumor Microenvironment
VEGF	Vascular Endothelial Growth Factor

6.1 Background

Cancer treatment across multiple solid and hematologic malignancies has been revolutionized with the approval of *checkpoint inhibitor therapy (CIT)* and *adoptive cell therapy (ACT)*. However, clinical benefit is derived in only a subset of patients. The next frontier in cancer immunotherapy is to unveil the innate and/or adaptive mechanisms of immune resistance that limit the efficacy of therapy in the majority of cancer patients [1]. A prevailing phenomenon observed in immune resistant cancers is an absent dialog between the cancer and immune cells [2]. This occurs in the context of “immune silent” [3, 4] and, even perhaps, “immune-excluded” tumors [2, 5, 6]. The latter are characterized by T cells restricted to the periphery of cancer nests. Since the activated T cells do not come in direct contact with the cancer cells, CIT is rendered immaterial unless the *tumor microenvironment (TME)* can be reprogrammed to facilitate T cell homing and infiltration [7].

Similarly, ACT may be affected by immune exclusion processes that limit direct T cell contact with targeted antigen-expressing cancer cells. More than two decades ago, Pockaj et al. [8] observed that in a great proportion of cases, adoptively

transferred *tumor infiltrating lymphocytes (TIL)* labeled with radioactive ^{111}In dium, which facilitates their tracking in vivo, did not localize to the targeted metastatic tumor sites. None of the patients in whom TILs did not traffic to the metastatic lesions experienced tumor regression suggesting that TIL localization to the tumor is an absolute requirement for the success of ACT. Furthermore, even successful homing of TILs to the tumor site was not entirely sufficient as only a proportion of patients responded to therapy even under this circumstance underlining the principle that cancer immune resistance results from distinct biologic processes [9]. We observed in patients with melanoma that response to TIL therapy is associated with the local expression of CXCR3 and CCR5-ligand chemokines capable of recruiting activated T cells to the metastatic site [10]. These observations highlight the multifactorial requirements needed to achieve an effective anti-tumor immune response [8, 10]. One may hypothesize that in the case of successful trafficking of TILs to the target lesions without accompanying tumor regression, immune exclusion mechanisms may have prevented the appropriately homed T cells from infiltrating the tumor nest and employing their full effector functions. Such hypothesis, however, has never been tested.

While it may be intuitive to attribute the silent immune landscape to a lack of tumor cell immunogenicity due to low tumor mutational burden [11, 12] and/or loss of the antigen processing and presentation machinery [13], immune exclusion may represent a more complicated phenomenon. The presence of T cells at the periphery of tumor nests suggests that chemo-attraction recruits the T cells to the tumor microenvironment and an antigenic stimulus promotes their persistence. However, a barrier prevents the T cells from infiltrating the tumor nests and to engage in direct cytotoxic cancer cell killing. Various hypotheses suggesting mechanical and/or functional barriers (Table 6.1) have been raised but no definitive consensus has been reached about the clinical relevance that each of them may play in human cancers.

6.2 Immune Infiltration and the Continuum of Immune Surveillance

Immunohistochemical (IHC) documentation of CD8⁺ T cells infiltration of solid cancers is a favorable prognostic biomarker [14–16], whereas a high density of CD163⁺ *tumor associated macrophages (TAMs)* is associated with poor survival [17]. Moreover, high ratios of CD8⁺ T cells over CD68⁺ myeloid cells or CD163⁺ TAMs bear favorable predictive and prognostic implications in patients with various cancers [18, 19]. However, an in-depth understanding of the contribution of the presence of distinct immune cell subsets within the TME can be better interpreted when morphological description is combined with functional characterization [3, 20, 21]. Representative transcriptional patterns such as the *immunologic constant*

Table 6.1 Hypotheses raised to explain immune exclusion (N.B. mechanisms may not be mutually exclusive)

Mechanical barriers	<i>Physical impediment to a direct contact between T cells and cancer cells</i>	
<i>Stromal fibrosis</i>	Filaggrin and desmosomal proteins	[27]
	Endothelin B receptor	[53, 66, 67]
	Defects in physical interaction between immune cells and TME	[62]
	Transforming growth factor (TGF)- β -induced fibrosis	[26, 63, 64]
	Epithelial mesenchymal transition	[57–61]
<i>Vascular access</i>	Vascular endothelial growth factor (VEGF)	[68, 69]
Functional Barriers	<i>Biological or metabolic interactions between cancer, stromal and immune cells limiting migration, function, and/or survival of T cells</i>	
<i>Metabolic barriers</i>	Nutrient depletion by cancer cells	[125–127]
	Warburg and reverse Warburg effect (lacto-genesis)	[73, 76, 78, 79, 127]
	Metabolic reprogramming of immune cells	[48, 128]
	Altered lipid metabolism	[129]
	Hypoxia	[80–85]
	K ⁺ levels	[74, 75]
	Glutaminase-dependent metabolism	[130]
	Metabolic inhibitors	[125, 131–134]
	Cyclooxygenase and prostaglandin metabolism	[135–137]
<i>Soluble factors</i>	<i>Cytokine/chemokine gradients</i>	[28, 81, 82, 100, 101]
	VEGF- α -mediated immune suppression	[68, 69, 87, 89, 92]
	TGF- β	[26, 63, 93, 94]
	Tumor-associated immune and stromal infiltrate suppressive mechanisms	[76, 101, 138–144]
<i>Danger sensing</i>	Tolerogenic cell death/absent immunogenic cell death	[97–99]
	Adenosine signaling	[101–103, 105]
	TAM receptor tyrosine kinases	[6, 106–108, 145]
	“Don’t eat me” signals (CD47/ <i>signal regulatory protein (SIRP)</i> - α axis)	[70–72]
<i>Tumor cell-intrinsic signaling</i>	Tumor cell-intrinsic β -catenin/signaling	[54–56, 121, 122]
	Extended PI3K pathway signaling	[115–120]
	Tumor cell-intrinsic STAT-3 activation	[111–114]
	Tumor cell-intrinsic MAPK signaling	[16, 45]
Dynamic Barriers	<i>Interactions between cancer and T cells resulting in limited function</i>	
	Checkpoint receptor/ligand interactions	[46–48]

of rejection (ICR) [20, 22] and the *tumor inflammation signature (TIS)* [3] include evidence of the activation of *interferon (IFN)- γ* signaling, immune effector mechanisms and the expression of CCR5 and CXCR3-ligand chemokines. These immune effector signatures have been confirmed as independent prognostic biomarkers and predictors of response to various types of IO agents. The ICR has been shown to predict prolonged survival in several independent data sets of breast cancer [16, 23, 24] and other cancers (Roeland' et al. *manuscript submitted*). Thus, the ICR has been applied to sub-classify cancers according to degree of immune activation in association with a favorable clinical connotation [7]. Moreover, the ICR was observed to be a predictor of immune responsiveness to the systemic administration of human recombinant *interleukin (IL)-2* [21] and to ACT with TILs [10]. The expression of the ICR signature is consistently accompanied by the expression of genes associated with immune regulatory function that have been implied to determine immune resistance [7]. These include transcripts representative of regulatory T cell and myeloid suppressor cell presence, and activation of the IL-23-Th17 axis, the *phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)- γ* pathway, the checkpoint cluster, and the IDO/NOS metabolic immune suppressors [7]. In addition, a transcriptional pattern representative of ICD was found to be strongly associated with the ICR suggesting a leading role for ICD as a determinant of immune activation. The congregation of immune effector and immune regulatory mechanisms within the same cancer landscape suggests that an evolutionary balance is required for the survival of antigenic tumors in the immune-competent host that depends on offsetting immunogenicity with compensatory mechanisms of tolerance [7]. In this case, resistance of tumors to IO agents and in particular CIT, maybe due to the multiple immune suppressive forces that operate within the TME; this may explain why targeting a single pathway with monotherapy by CIT may not suffice to induce tumor rejection [7, 25]. Further, discussion about the biology of immune active cancers and related *compensatory immune resistance* is beyond the scope of this chapter as it was discussed elsewhere [1, 7] and is the subject of another chapter in this book (Bedognetti et al., *The biology of immune active cancers and their regulatory mechanisms*).

6.3 Prevalence of the Immune Excluded Phenotype

The phenomenon of immune exclusion has been described by several investigators [2, 5, 6, 26–32]. Galon et al. [14] observed that the infiltration of CD8 T lymphocytes within tumor nests combined with their peri-tumoral presence is the principal parameter that prognosticates improved survival of patients with colorectal cancer with higher accuracy than the classical TNM staging. This observation was recently validated by an international, multi-institutional study [15]. In contrast, the limited presence of T cells at the periphery of tumor nests without intra-tumoral infiltration, which corresponds to what we define as “*immune exclusion*,” did not bear any significant impact on survival [14, 15, 33, 34]. This

observation powerfully suggests that immune exclusion represents a functionally distinct biological entity with distinct clinical implication.

It is well accepted that in most cases, individual cancers segregate according to three distinct landscapes: immune active, immune silent, and immune excluded [28] although occasionally a combination of these landscapes can be observed within the same tumor [35]. The prevalence of each landscape in cancers of different histology remains undetermined. This information may be critical for the stratification of patients during clinical trials [30, 36–40]. A recent study systematically measured by IHC the pattern of immune infiltration (“*topography*”) of 965 histological tissue slides from 177 patients bearing cancers of different histology [28]. The tissue specimens were categorized according to the number of cells per mm^2 in three spatial compartments: *outer invasive margin* (0–500 μm outside the tumor invasion front), *inner invasive margin* (0–500 μm inside the tumor invasion front), and in the tumor core (>500 μm inside the invasion front). Preliminary analysis demonstrated that there was a strong correlation between the infiltration of the tumor core and the inner invasive margin. Thus, these two categories were combined to define immune active or “*hot*” tumors. Whereas, tumors with high immune cell density in the outer invasive margin and low density in the core were defined as immune “*excluded*” tumors (parenthetically, this is the most precise definition of immune exclusion that we have encountered so far). Low density in all compartments characterized “*cold*” tumors. Distinct cutoff values were used for different immune cell types including CD3^+ (363 cells/ mm^2), CD8^+ (295 cells/ mm^2), Foxp3^+ (62 cells/ mm^2), PD1^+ (6 cells/ mm^2), CD68^+ (310 cells/ mm^2), and CD163^+ cells (559 cells/ mm^2). Distinct tumor types displayed remarkably different patterns of immune infiltration. Cancers deemed sensitive to IO agents and in particular CIT, such as melanoma, lung adenocarcinoma, lung squamous cell carcinoma, and *head and neck squamous cell carcinoma (HNSC)*, displayed a high frequency of simultaneously CD3 -hot, CD8 -hot, and PD1 -hot tumors whereas colorectal cancers presented most frequently with an immune excluded phenotype. With bivariate immune cell analysis, several reciprocally exclusive behaviors were observed: while CD3 and CD8 -expressing T cell infiltrates were generally concordant, inverse correlations were observed between CD8 and Foxp3 T cells and CD68 (TAMs) infiltrates. The critical observation of this study suggested that bivariate classifications including several immune cellular components are most likely to provide clinically relevant information. An interesting example was provided by Tsujikawa et al. [30] wherein HNSC were segregated according to the presence of oncogenic *human papilloma virus (HPV)* known to be associated with an immunogenic gene signature [41, 42]. The HPV positive cases were sub-classified into tumors that were predominantly expressing only lymphoid signatures and tumors that included a mixture of lymphoid and myeloid cell signatures. Interestingly, in the case of HPV-associated cancers, the worst prognostic connotation was associated with the presence of a strong myeloid infiltrate that was independent of the lymphoid infiltrate [30]. This study was performed on tissue microarrays that did not allow spatial discrimination of invasive margins versus tumor core assessment. Thus, it remains unknown whether lymphoid to myeloid ratios are also affected by spatial

distribution according to the “*geocentric*” description of cancer immune exclusion presented in this chapter. A critical relevant observation of this study was the realization that immune excluded cancers are much more prevalent across cancer histologies than generally perceived. This observation is important because it may suggest that an opportunity for the design of next-generation IO agents is to determine the prevalent mechanisms of immune exclusion and address them to expand the effectiveness of IO approaches to cancer in which immunity is relevant but literally plays only a “*peripheral*” role.

We propose that the biology of immune excluded tumors is partially similar to that of the immune active as compared to the immune silent phenotype. This hypothesis is based on the premise that chemo-attraction can recruit the T cells to the tumor periphery and that some immunogenic stimulus can preserve their persistence. The question remains, however, whether additional mechanisms are required to stimulate immune infiltration that are present in hot tumors but not in immune excluded (active recruitment). Conversely, immune exclusion could be determined by chemo-repulsive mechanisms that are not present in hot tumors.

Limited observations in HNSCC suggest that immune excluded cancers are transcriptionally indistinguishable from the immune active ones. We applied the hallmark ICR signature to segregate HNSCC samples and observed that both immune excluded and immune infiltrated tumors demonstrated a full display of an immune effector Th1-like polarization of T cells. Consistent with previous observations [16, 24, 43–45], the Th1 polarization was combined with the presence of immune-suppressive mechanisms (*Pai SI. et al., ASCO Abstract # 6052*). Thus, the distinction between the immune excluded versus the immune active phenotypes is primarily the spatial resolution and localization of immune cell populations and these two immune phenotypes cannot be distinguished solely based on functional signatures [28]. The ICR signature includes Th1 polarization markers such as IFN- γ -related transcripts, granzyme, and perforin that are detectable upon cognate activation of T cells by antigen exposure. Thus, it appears that in the immune excluded HNSCCs studied, CD8 T cells come into contact with and recognize cancer cells at the border of the tumor nest but some functional barrier prevents their infiltration into the tumor. However, this conclusion may not apply to all cancers. In melanoma and ovarian cancer, we observed that the expression of genes regulating physical barriers was in some cases inversely correlated with the ICR signature [27]. This observation suggests that when mechanical barriers prevent T cell infiltration, no direct contact occurs between cancer cells and T cells and, therefore, no activation of immune effector gene signatures can be observed. Since this study did not include extensive histological examination of the samples, it remains unknown if the inverse correlation relates to immune silent or immune excluded cancers.

Functional barriers may not be present in baseline conditions but they may be *induced* only when a contact occurs between T cells and cancer cells preventing subsequent infiltration into tumor nests [46–48]. As discussed later, we refer to this concept as dynamic barriers.

Thus, we propose that mechanisms potentially responsible for immune exclusion could be segregated into three functional categories according to the relevance of (1) mechanical barriers that pose a physical impediment which prevents the contact between T cells and cancer cells, (2) functional barriers that consist of steady-state biological and/or metabolic interactions between cancer, stromal, and immune cells which limit the migration, function and/or survival of T cells, and, lastly, (3) dynamic barriers that are induced upon the interaction between cancer and immune cells but then prevents further T cell recruitment, migration, and/or survival.

Distinguishing these three mechanisms of immune exclusion has important implications in guiding novel IO therapeutics aimed at overcoming immune resistance. For example, a lack of chemo-attraction of T cells to the tumor site may not be the rate-limiting factor in achieving a successful anti-tumor immune response in immune excluded tumors as compared to immune silent cancers. Thus, immune excluded tumors may require a completely different therapeutic approach compared to immune silent cancers as suggested by the previously described ¹¹¹Indium-labeled TIL study [8]. Furthermore, understanding these mechanisms of immune exclusion has critical therapeutic implications in the successful application of ACT of solid tumors.

In the case of functional and dynamic barriers, the implication that some albeit aborted interactions occur between cancer and T cells at the periphery of the tumor nests presents the optimistic opportunity that interference with functional barriers by genetically reprogramming T cell function may overcome these immune exclusion mechanisms [49–51]. An example of such therapeutic opportunity is represented by the regulation of PD1 expression on T cells by genetic knockout or conditional knockdown to overcome the interaction with PDL1 molecules expressed either by tumor cells or TAMs at the periphery of tumor nests [46] or the constitutive or conditional expression of factors aimed at counteracting immune suppressive signals [52].

6.4 Mechanisms of Immune Exclusion

The biology establishing immune exclusion, defined as the presence of T cell lining the outer margins of tumor nests [28], remains unknown. In particular, it is unknown whether a prominent mechanism is responsible for most cases rather than this well-defined phenotype resulting from the convergence of multiple inconsistently and unpredictably occurring biological disruptions that in distinct ways hamper T cell infiltration within the tumors nests. Here, we separate potential mechanisms according to main categories (Table 6.1).

6.4.1 Mechanical Barriers

Physical impediment preventing contact between T cells and cancer cells

In two independent cohorts that included 114 metastatic melanoma and 186 ovarian cancer samples, we identified eight genes that encode for proteins with mechanical barrier function. The expression of those genes was inversely correlated with that of the Th1-like ICR immune signature. Their expression was also associated with worse prognosis in patients with melanoma [27]. Among them, the expression of the desmosomal protein *dystonin (DST)* marked cancers lacking in absolute the expression of the Th1 immune signature suggesting that no interactions were present in these tumors between T cells and cancer cells. The other seven genes demarcated a set of cancers characterized by reduced and variable expression of the ICR signature suggesting that in this case some functional interactions were occurring. Importantly, expression of the latter barrier molecules occurred independently of the expression of endothelin receptor B and, in a mutually exclusive manner, with the activation of the β -catenin signaling pathway both reported to interfere with T cell infiltration into human cancers [53–56]. These observations suggest that several mechanisms of immune exclusion may shape the cancer immune landscapes and that such mechanisms may be at times mutually exclusive.

Interestingly, *DST* expression identified a subset of melanoma cases in which absolute absence of $CD8^+$ gene signatures was observed but this was not associated with decreased patient survival. This supports the concept that the absolute presence of T cells within a given TME is not a singular prognostic determinant and possibly the ratio between lymphoid over myeloid or other cellular infiltrate may better define prognostic significance [18, 19, 30]. This hypothesis, however, was not explored by this study. Limited IHC analyses performed on those samples validated the expression of the barrier molecules at the protein level. In addition, these limited IHC analyses suggested that the expression of protein that could function as physical barriers was predominantly observed in cancer cells. The expression of these markers was associated morphologically with the cold or immune silent TME and this observation is also consistent with the exclusion of transcriptional ICR signatures suggesting that mechanical barriers are more likely to be relevant to the immune silent than the immune excluded landscape. Conversely, the true immune excluded landscape is more likely due to functional rather than physical barriers. Clearly more extensive analyses combining histological with functional methods will be needed to test this hypothesis.

Various other mechanisms are likely to play a role in influencing T cell migration. For instance, epithelial to mesenchymal transition altering the stromal composition has been accounted for in the development of a mechanical barrier [27, 57–59]. This in turn is largely mediated by *transforming growth factor (TGF)- β* activity [60, 61].

Epithelial to mesenchymal transition is not the only way in which TGF activity may lead to fibrosis mediated in cancer [26, 62, 63]. This pleiotropic cytokine is indeed essential for the induction of fibrotic responses through a cross talk with

extracellular matrix components during organic tissue regeneration. Moreover, TGF- β is a critical component of the TME mediating resistance to immune surveillance mechanism through several direct and indirect mechanisms that restricts T cell trafficking and function by acting on the modulation of various cell populations contributing to the development of mesenchymal stromal cells [26, 64] and *cancer-associated fibroblasts (CAF)* [63]. The latter bear several immune regulatory properties beyond the generation of fibrous material contributing simultaneously to the development of both physical and functional barriers. For this reason, TGF- β is considered a promising therapeutic target particular for immune silent cancers. Its role in determining specifically immune exclusion remains, however, indeterminate [65].

Another mechanism of physical exclusion of T cells could be related to limited vascularization; it can be presumed that less vascularized tumors may be less prone to immune infiltration. In addition, various endothelial receptors may play either facilitator or inhibitor roles by mediating immune cell translocation into the extravascular space [53, 66, 67]. Independent of specific receptors mediating T cell transposition across the vascular structures, *vascular endothelia growth factors (VEGF)* seems to play a prominent role by mediating not only the access of immune cells into tumors but also their function [68, 69]. The latter mechanism probably plays a major role in determining immune resistance to IO agents and, therefore, similarly to TGF mediates the activation of both functional as well as mechanical barriers.

6.4.2 Functional Barriers

Pre-existing biological and/or metabolic interactions between cancer, stromal, and immune cells limit the migration, function, and/or survival of T cells

The mechanisms limiting immune infiltration through adaptive or innate interactions are extensive. Moreover, other mechanisms may be indirectly determining immune exclusion by dampening the production of pro-inflammatory and/or chemo-attractive signals. For instance, innate immune cells may less efficiently process and present apoptotic cancer cells for immune activation because of the presence of “*don't eat me signals*” [70–72] and/or other tolerogenic mechanisms in which cancer cells may die, as discussed later.

It is important to emphasize that, to our knowledge, there is no integrated view about how various mechanisms may relate to each other in determining immune exclusion in human cancers either from a causative standpoint or through converging biologic routes. Moreover, the prevalence of distinct mechanisms and their weight in shaping the TME of human cancer is unclear and it is unknown whether this plethora reflects an equal distribution of various ways that immune exclusion may be randomly distributed across different cancer types or a predominant mechanism is responsible for most cases while others may only occasionally play a significant role. In other words, the longstanding debate of whether cancer is one disease or rather a mosaic of diverse diseases is best exemplified by the chaotic

models used to describe immune exclusion. For instance, Schwartz et al. [73] suggested that most of the hallmarks of cancer can be attributed to the Warburg effect according to which, even in aerobic conditions, cancer cells favor glycolysis over oxidative phosphorylation. According to this view, the metabolic impairment of oxidative phosphorylation triggers a cascade of events that include the fractal shape of tumors, the secretion of collagen by CAFs, the production of lactic acid that elicits an acidic TME with direct and indirect effects on innate and adaptive immune functions. Furthermore, this can induce increased intracellular alkalosis in cancer cells and alteration of mitochondrial function leading to dysregulation of the ion concentration in the TME with its increasingly recognized effects on T cell function [74, 75]. Whether this hypothesis or modification around it reverses the Warburg effect whereby cancer cells induce aerobic glycolysis in neighboring CAFs [76] is debatable as discussed by Xu et al. [77]. However, its weight is not as important as the general concept suggested by the authors that efforts should be placed to identify convergent views regarding this otherwise chaotic portrait of immune exclusion.

An integrated approach to resolve the enigma of immune exclusion should include the simultaneous analysis of biomarkers representative of distinct mechanisms of immune exclusion in the same tumor samples. This approach can identify associations that may have similar upstream mechanistic determinants. The analysis may prove even more effective if a topographical analysis will be performed surveying the centripetal gradients within individual tumor nests. To our knowledge, such comprehensive analysis has never been done nor is currently being entertained by any investigation on human cancer samples and we encourage such efforts as part of the systematic and integrated approach to the solution of the quandary of cancer immune resistance [1]. A better understanding of the dominant mechanisms leading to immune exclusion and their primary causation may lead to a better rationale for drug development and for the selection of sound combination therapies.

A comprehensive discussion about individual theories proposed to explain a functional basis for immune exclusion is beyond the purpose of this chapter and we refer to the content arranged into sub-categories in Table 6.1. Here, we are limiting the discussion to principles that reflect similar biologic facets. We propose that functional barriers could be categorized into those determined by metabolic alterations specific to the TME as exemplified by the Warburg effect [73, 78, 79] as discussed previously.

A separate role may be played by hypoxia [80]. In fact, most cancers thrive in hypoxic conditions due to suboptimal vascularization. This in turn, has been shown to lead to profound depression of T cell function that is sufficient on its own to lead to immune exclusion [81–85]. Related to hypoxia is the complex and pleiotropic role that VEGF family members play in the TME by regulating lymph angiogenesis. It is likely that hypoxia is at least a cause of increased VEGF family members including VEGF-A, -C, and -D presence in the TME [86]. Although intuitively VEGF should increase lymphocyte infiltration in tumors due to increased angiogenesis, most observations refute this concept demonstrating that indeed anti-angiogenic therapies enhance immune infiltration of T cells [69, 87–89].

Indeed VEGF has been shown to mediate direct and indirect immune suppression through a series of distinct mechanisms [68] once again pointing out that the border between physical and functional barriers is not always well demarcated and that often, the same pathways may lead to contrasting effects due to the pleiotropic properties of the cells involved and the factors that they secrete. Thus, like for the Warburg effect, hypoxia may represent a principal component of immune exclusion mediating directly and indirectly immune infiltration and function. To complicate things, it has been shown that VEGF family members can be overexpressed constitutively by cancer cells independent of hypoxic conditions that stimulate their production [90–92].

An increasing gradient of repulsing signals is best exemplified by TGF- β . As previously mentioned, this extremely pleiotropic factor exercises effects on the TME way beyond the stimulation of the production of fibrotic material [26, 63]. Beyond its effects on stromal cells including CAFs, TGF- β acts directly on immune cells and in particular CD4⁺ T cells [93, 94].

Chemo-attraction (and lack thereof) is a factor that regulates immune infiltration. While several chemokines have been implicated in the recruitment of T cells and other immune effector cells to the TME, it appears that CXCR-3 and CCR-5 ligand chemokines play a dominant role in immune surveillance and they are most significantly correlated with CD8 T cell infiltration as part of the ICR signature [10, 20, 22, 95]. The central role that these two families of cytokines play in immune surveillance has been clearly established for both immune active and immune silent cancer as they are tightly correlated to these two phenotypes [7]. The question remains, however, about the role that they may play in the context of immune excluded tumors. Intuitively, these or other T cell attracting chemokines should be present to attract T cells at the periphery of tumor nests, although this presumption has never been tested. It is, therefore, possible that for unknown reasons an abrupt reduction in the gradient of chemo-attraction from the center to the periphery, such as expression of CXCR3 and CCR5 associated chemokines, may reduce the chemo-attractive propulsion that occurs at the periphery. For instance, it was recently suggested that in melanoma chemokine expression is modulated by cancer cell-intrinsic pathways, such as microphthalmia-associated transcription factor, whose expression is associated with that of CXCL-10 [96]. Alternatively, while the expression of these chemokines is stably maintained within the concentric circles of the tumor nest, additional overpowering repulsive mechanisms emanated from the center may counterbalance the attractive signals gradually limiting the progression of T cells.

Evolving patterns of cell death from the germinal center to the periphery may create a gradient of increasing chemo-attraction. Possibly, cancer cells may rapidly dedifferentiate from a stem cell-like core to a degenerate progeny at the periphery prone to **Immunogenic Cell Death (ICD)** [97–99]. This could be tested by surface expression of calreticulin or other ICD markers [100]. Alternatively, cancer cell death occurs only at the periphery while the “germinal” centers continue to actively proliferate (this could be tested using proliferation markers). Thus, **Damage Associated Molecular Patterns (DAMPs)** may be present only at the periphery. To

our knowledge, this possibility has never been entertained and remains only speculative. A more likely possibility is that various mechanisms affecting the processing of stressed or dying cells may play a significant role by dampening pro-inflammatory signals. Several of these mechanisms include adenosine signaling [101–105], TAM receptor tyrosine kinases [6, 106–108] and the “*don’t eat me*” signals through the CD47/*signal regulatory protein (SIRP)- α* interactions [70–72]. Extracellular adenosine is an almost ubiquitous component of tissues exposed to stress. It is generated in response to pro-inflammatory conditions and in particular hypoxia [105] when stressed cells release ATP. The latter is then degraded by CD39 into AMP and subsequently turned into adenosine by CD73. Adenosine in turn interacts with several G-protein-coupled receptors almost ubiquitously expressed on the surface of most immune cells. These receptors promote anti-inflammatory responses as described comprehensively by Young A et al. [104]. Similarly, TAM receptors (Tyro3, AXL and Mertk) are tyrosine kinases that orchestrated a prominent regulatory role particular on innate immune cell functions by promoting the phagocytosis of apoptotic cells and inhibiting inflammation [109]. Through this process, TAM receptors inhibit natural dendritic cell and macrophage maturation signals [109]. Consequently, their targeting promotes the activation of immune responses against cancer as well exemplified by the potentiation of radiation effects by the inhibition of Mertk [107]. Similarly, to TAM receptors, other cancer cell/immune checkpoint interactions may occur as well exemplified by the CD47/SIRP- α interactions [70–72, 110]. Although CD47 interacts with its ligand expressed by cells of the myeloid lineage by inhibiting phagocytosis and clearance of dying cells, it appears that it also plays a direct anti-inflammatory role by regulating the activation of innate immune effector cells [70]. Thus, it could be considered a cell surface checkpoint molecule target for immunotherapy [71, 72].

A final category of potential mechanisms of immune exclusion is represented by the direct effect that cancer-intrinsic signaling pathways can play in modulating chemo-attractive signals and immune modulatory responses. Among them, the role played by tumor cell-intrinsic *signal transducer and activator of Transcription (STAT)-3* [111–114] and *phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)* [115–120] signaling have been extensively described. In addition, the contribution of alterations in β -catenin [54–56, 121, 122] and MAPK [16, 45] signaling have been described. Interestingly, both PI3K and STAT-3 signaling are prominent features of myeloid cell activation [113, 114, 123, 124] and, therefore, their role in immune modulation is often confused with the genuine alteration of intrinsic cancer pathways. Interestingly, most studies referring to dysregulation of the PI3K pathway refer to mutations of genes involved in cancer cell signaling. However, the same pathway is critical in the activation of myeloid suppressor dendritic cell function downstream of TAM receptor signaling [107, 108]. This functional signature is totally independent of the cancer cells mutational status and it is purely related to immune cell functions determined by other signaling mechanisms. For instance, we noticed that transcriptional signatures associated to activation of the PI3K pathway were most frequently observed in immune active cancers [7, 25] and this activation was most likely due to the myeloid cells, while in those tumors no

specific somatic mutations relevant to cancer cell biology were noted particularly related to STAT3 and PI3K. Conversely, β -catenin and MAPK activation have been associated with mutational status of genes within the respective pathways and are, therefore, to be considered purely related to activation of cancer cell-intrinsic pathways [43, 44]. We believe, however, that both mechanisms are not likely to be relevant to immune exclusion as both are tightly associated with the immune silent cancer phenotype and the absolute lack of expression of the ICR signature [7]. We recently validated this observation in a set of 9,282 tumor samples in a Pan-cancer analysis of cancer of 31 different histologies (Roelands J. et al., submitted). Yet, no specific analysis attempting to match morphologic features with transcriptional activation have been so far entertained to verify this assumption.

6.4.3 Dynamic Barriers

Biological interactions between cancer and T cells that result in limited function

As previously described, the regulatory interactions that limit T cell infiltration into tumor nests may not be present in baseline conditions but develop as a consequence of the first encounter between T cells and cancer cells. This is well exemplified by the inducible activation of PDL-1 in response to IFN- γ signaling. It has been observed in the context of head and neck squamous cell carcinoma that *programmed cell death protein-1 (PD-1)* is observable in functionally anergic PD-1 expressing TILs at the periphery of tumor nests. The latter are flanked by tumors and/or CD68⁺ TAMs that express *programmed death ligand-1 (PDL-1)*. This observation suggests that a functional cross-check occurs dynamically at the initial encounter between T cell and cancer cells possibly triggered by the production of IFN- γ by T cells upon exposure to cognate stimulation. This in turn, induces expression of PDL1 by either cancer or TAMs that serves as a negative feedback loop to limit the function of CD4⁺ and CD8⁺ T cells [46] (Fig. 6.1). Similarly, close dynamic interactions between PD-1⁺ CD8⁺ T cells and PD-L1 expression cancer cells, and/or CD68⁺ TAMs were reported in human papillomavirus-associated HNSCC [47]. Finally, a cross talk between T cells and dendritic cells expressing PDL1 in response to IFN- γ and IL-12 stimulation plays a critical role in modulating immune responsiveness [48]. Whether these dynamic interactions may be at the basis of immune exclusion has been sufficiently investigated but we believe that future studies should include extensive analyses integrating transcriptional with morphological description of cancer immune phenotypes.

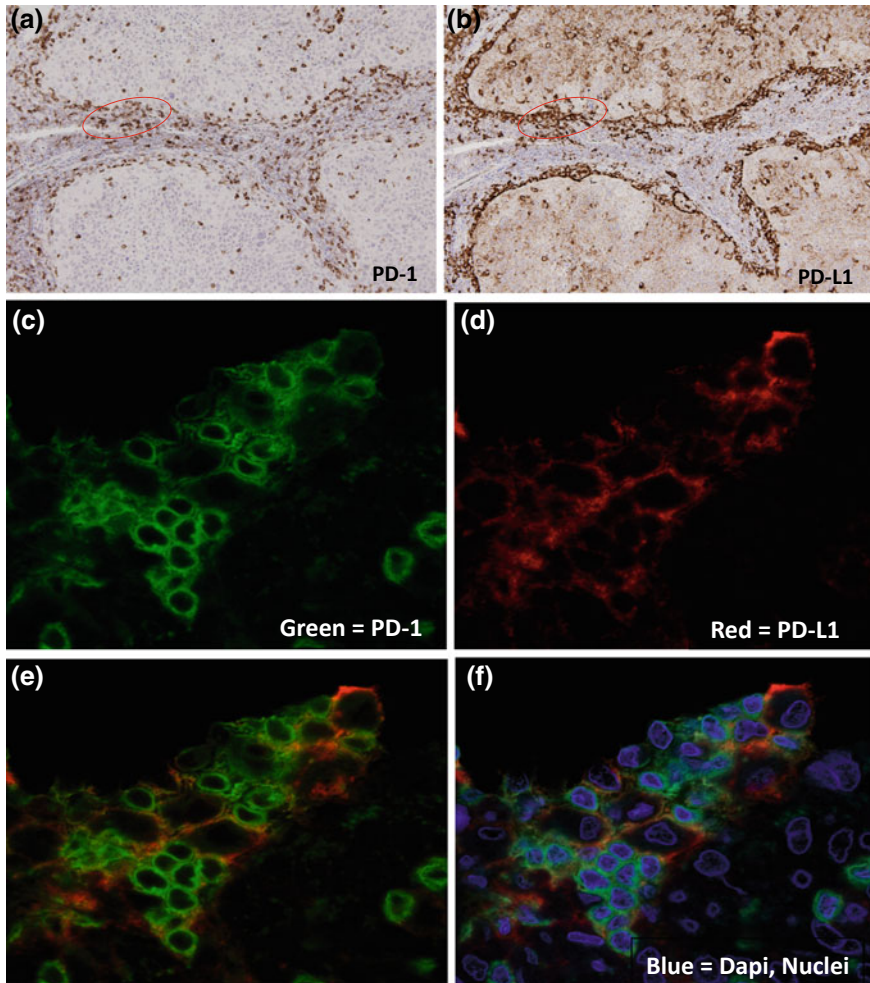


Fig. 6.1 The Paradox of Immune Exclusion—Example of distinct theories that may explain the confined presence of T cells at the periphery of immune excluded cancer nests. **a.** Single immunohistochemical staining of PD-1 receptor on T cells in HNSCCs. Although there is some T cell infiltration into the tumor nests, the majority of the activated T cells remain in the tumor stroma and in the periphery of the tumor nest depicting immune exclusion. **b.** Single immunohistochemical staining of PD-L1 on the tumor and/or immune cells on the same HNSCCs cut in serial section. There is strong PD-L1 expression in the periphery of the tumor nests as well as on tumor infiltrating TAMs. **c.** Single immunofluorescent staining of PD-1 receptor. This is a high magnification of the area circled in red in Panel A. **d.** Single immunofluorescent staining of PD-L1. This is a high magnification of the area circled in Panel B. **e.** Dual immunofluorescent staining of PD-1 and PD-L1 with areas of overlap depicted as orange. **f.** Dual immunofluorescent staining of PD-1 and PD-L1 with blue DAPI staining of single cell nuclei

6.5 Clinical Implications

Improved understanding of the mechanisms that drive immune exclusion has important clinical implications in the development of novel therapeutic strategies aimed to overcome immune resistance against IO agents, including ACT. It remains to be clarified whether a predominant biology is at the basis of most immune excluded cases. Future studies may reveal that a complex combination of mechanical, functional, and dynamic barriers may shape the immune biology of individual tumors attesting for the need for more in-depth precision medicine-based molecular and histological characterization to be implemented for the selection of appropriate monotherapy or combination therapeutics.

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