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EVOLVING THERAPIES (R BUKOWSKI, SECTION EDITOR)

Cancer Clonal Theory, Immune Escape, and Their Evolving Roles in Cancer Multi-Agent Therapeutics

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Abstract
Purpose of Review The knowledge base of malignant cell growth and resulting targets is rapidly increasing every day. Clonal theory is essential to understand the changes required for a cell to become malignant. These changes are then clues to therapeutic intervention strategies. Immune system optimization is a critical piece to find, recognize, and eliminate all cancer cells from the host. Only by administering (1) multiple therapies that counteract the cancer cell's mutational and externally induced survival traits and (2) by augmenting the immune system to combat immune suppression processes and by enhancing specific tumor trait recognition can cancer begin to be treated with a truly targeted focus.
Recent findings Since the sequencing of the human genome during the 1990s, steady progress in understanding genetic alterations and gene product functions are being unraveled. In cancer, this is proceeding very fast and demonstrates that genetic mutations occur very rapidly to allow for selection of survival traits within various cancer clones. Hundreds of mutations have been identified in single individual cancers, but spread across many clones in the patient's body. Precision oncology will require accurate measurement of these cancer survival-benefiting mutations to develop strategies for effective therapy. Inhibiting these cellular mechanisms is a first step, but these malignant cells need to be eliminated by the host's mechanisms, which we are learning to direct more specifically.
Summary Cancer is one of the most complicated cellular aberrations humans have encountered. Rapidly developing significant survival traits require prompt, repeated, and total body measurements of these attributes to effectively develop multi-agent treatment of the individual's malignancy. Focused drug development to inhibit these beneficial mutations is critical to slowing cancer cell growth and, perhaps, triggering apoptosis. In many cases, activation and targeting of the immune system to kill the remaining malignant cells is essential to a cure.

Keywords Immune escape · Cancer clonal theory · Immune therapies · Liquid biopsy

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Introduction

Since its first proposal by Peter Nowell in 1976 [1•], the clonal evolution model of cancer development has become increasingly widely accepted. Importantly, cancer is a dynamic and evolutionary disease, the complexity of which must be appreciated for treatment strategies to be truly effective. Although there have been significant advances in cancer treatment in recent years, we believe that the future of precision medicine will require multiple therapies that attack individual clones containing a mosaic of genetic and induced survival-enhancing traits. A recent study that assessed over 7000 tumor

samples demonstrated that there were nearly 5 million mutations in the tissue population, corresponding to an average of over 700 mutations per sample [2•]. Cancer-associated mutations appear in coding regions and non-coding regions, such as promotors and introns [3•]. It must be assumed that many of these mutations have no effect on cellular function and may even have detrimental effects leading to clone death, while some others will have survival advantage ramifications. These beneficial survival mutations appear to be in several distinct functional areas of the genome like those that control (1) growth promotion, (2) growth inhibition and apoptosis, and (3) nutrition and energy processing. Measuring these cellular alterations is critical to "precision oncology" and drug development target determination. The clones themselves have some similar inherited traits from parent cells, but these daughter cells can develop new, synergistic mutations conferring novel and more beneficial survival qualities. To eliminate cancer will require multiple therapeutics that interfere with many, perhaps all, survival trait functions within each of the surviving clones. This strategy can lead to disruption of the survival mechanisms to stop growth, induce apoptosis, or other means of cellular death such as inhibiting metabolic pathways. In fact, it may turn out that a patient will be prescribed 7–32 or more medications to counter clonal disease mutations allowing internal cancer mechanisms to induce cell death, while mediating immune system function to eradicate disease in first-line therapy when these survival traits are at the recognizable minimum.

Cancer Clonal Theory

The Clonal Evolution Theory has been validated [4•, 5•], but rigorous study and understanding of the theory is needed to justify its incorporation into practice. In the current model of the theory, a phenotypically normal cell undergoes some genetic (or epigenetic) change that induces a self-renewal pathway and acts as a driver mutation, starting the cascade of branching tumor development [1•]. We have described this not as just evolution in a Darwinian sense but as a "revolution" where mutations are occurring very rapidly [6•].

Once a beneficial mutation like this arises, there needs to be a stabilization of the cell's internal machinery as well as its external local environment. Further mutations that allow for telomere stabilization and angiogenesis provide the initial necessary factors for long-term tumor survival [7•]. Survival, however, also means the ability to resist killing by the active innate and adaptive immune systems. Early mutated cells may be eliminated by the host immune system. In some cases, abnormal cellular growth can keep pace with immune system killing, resulting in an equilibrium between growth and death [8•]. A critical next step to malignancy is the development of one or more methods to thwart the host immune system processes. Some tumors have been shown to develop immunosuppressive extracellular ligands (inappropriate checkpoint expression by cancer cells) that allow the tumor to develop further [9], which has prompted the use of checkpoint-directed monoclonal antibodies [10, 11] (see Table 1).

The mutations developed prior to clinical cancer are classified into four categories. First, "driver" mutations further the progression of disease by promoting growth or inhibiting apoptosis or growth cessation. Secondly, "passenger" mutations do not initially directly affect tumor stability, but come into play later in development. Passenger mutations become significant when other loci have been mutated that normally interact with the passenger mutation or when a therapy is applied that highlights the passenger mutation, where it can then add a survival advantage. Thirdly, mutations can be deleterious and cause immediate cell death, which is presumably common. Lastly, mutations can affect genes that are essential to DNA integrity. These mutations, termed "mutators," increase the rate of mutations and further tumor development by expanding the possible mutations [15, 16]. Mutations to the tumor's interactions with its local environment also play an important role [17]. These mutations often allow for rapid continuous cell growth and division by uptake of required nutrients and energy sources.

The changes in tumor genetics may not be solely dependent on the DNA sequence, however. It has been proposed that there may be significant epigenetic changes in the tumor cell that result in disease development [18]. Changes that affect any portion of the cancer environment can play a huge role in progression of disease as well. The Cancer Ecosystem, as defined by Greaves and Maley, is categorized into three sections: (1) Systemic Regulators (i.e., hormones, growth factors, immune/inflammatory response cells, and cytokines), (2) Local Regulators (i.e., O_2, metabolism, nutrients, and cell-cell and cell-stroma/matrix interactions), and (3) Architectural Constraints (i.e., physical compartments, basement membranes, restricted niches) [19•].

The genetic solutions to the tumor's environmental pressure take a significant amount of time and several rounds of mutation, growth, and death to be uncovered. Moreover, once a mutation that counteracts one of the constraints of the microenvironment is achieved, a new, more adapted survival characteristic becomes incorporated in the clone's survival repertoire. Clones that were less successful in acquiring beneficial traits may survive, but not optimally compared to other clones. Once a specific selection pressure occurs, the survival growth rate of different clones may change. The length of time needed to develop disease is seen in the significant concentration of cancer in elderly patients greater than 60 years of age [20]. Furthermore, there may be tumor-suppressive pathways that are active until old age, at which point they can lose their effectiveness. The idea of clonal mutation, growth, and selection is observed in the replication times of cells as well. On

Table 1 Checkpoint inhibitors currently available and approved by the FDA [12–14]

Generic name, trade name mechanism of action	Indication for approval
Avelumab, BAVENCIO PD-L1 blocking antibody	Metastatic Merkel cell carcinoma - Treatment of adults and pediatric patients 12 years and older
Atezolizumab TECENTRIQ PD-L1 blocking antibody	Metastatic non-small-cell lung cancer (NSCLC) - The treatment of patients with metastatic non-small-cell lung cancer (NSCLC) who have disease progression during or following platinum-containing therapy Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy for these aberrations
	Locally advanced or metastatic urothelial carcinoma - After progression within 12 months of neo-adjuvant or adjuvant with a platinum regimen - Disease progression during or following treatment with platinum-containing regimen
Ipilimumab YERVOY CTLA-4 blocking antibody	Adjuvant treatment of patients with cutaneous melanoma with pathologic involvement of regional lymph
	Unresectable or metastatic melanoma - In combination with nivolumab
	Adjuvant treatment of patients with cutaneous melanoma with pathologic involvement of regional lymph nodes
Nivolumab, OPDIVO, PD-1 blocking antibody	Locally advanced or metastatic urothelial carcinoma - After progression within 12 months of treatment with platinum-containing treatment
	Recurrent or metastatic squamous cell carcinoma of the head and neck (HNSCC) - With disease progression after platinum-based treatment
	Classical Hodgkin lymphoma (cHL) - Relapsed or progressed after autologous hematopoietic stem cell transplantation - Or three or more lines of systemic therapy + auto-HSCT
	Advanced renal cell carcinoma - Patients who have received prior anti-angiogenic therapy
	Metastatic non-small-cell lung cancer (NSCLC) - With progression on or after platinum-based chemotherapy
	Unresectable or metastatic melanoma - BRAF V600E wild-type or mutated as single agent
	Unresectable or metastatic melanoma I-n combination with ipilimumab
Pembrolizumab, KEYTRUDA PD-1 blocking antibody	Microsatellite instability-high cancer - Adult and pediatric patients with unresectable or metastatic, microsatellite instability high (MSI-H), or mismatch repair deficient
	Urothelial carcinoma - Locally advanced or metastatic urothelial carcinoma not eligible for cis-platinum containing chemotherapy
	Classical Hodgkin lymphoma (cHL) - Relapsed after three or more prior lines of therapy
	Non-small-cell lung cancer (NSCLC) - If tumor expresses PD-L1 as determined by an FDA-approved test - Single-agent first line, PD-L1 >50% - Single agent after prior platinum regimen PD-L1 >1% - In combination with premetrexed and carboplatin as first-line Rx
	Head and neck squamous cell carcinoma (HNSCC) - Recurrent or metastatic and progression after platinum-containing chemotherapy
	Melanoma - Including first-line PD-L1 >50%; single-agent PD-L1 >1% after first-line platinum-containing regimen
	Unresectable or metastatic melanoma - Disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor

average, it takes approximately 1–2 days for tumor cell doubling while it takes anywhere from 60 to 200 days for complete tumor size duplication [21]. This observation indicates that the vast majority of tumor cells die shortly after dividing and, therefore, do not contribute to overall tumor growth [22]. It is also important to note that many current cancer therapies are toxic to cancer cells, but unintentionally select for those cells that have acquired passenger mutations that provide for therapy resistance. These newly selected resistant cells follow a revolutionary trajectory and replicate to induce a relapse in the patient. While the environment exerts some selective pressures, cancer treatment is possibly the most potent selective force.

The new progression toward "precision medicine" has prompted physicians to obtain more tumor samples in the form of liquid biopsies. The idea behind the procedure is to obtain the genomic, transcriptomic (mRNA), and proteomic characterization of the tumor to select targeted therapies. Unfortunately, tumor biopsy samples that are subsets of the total cancer burden can lead to less than precise information from the non-biopsied clonal regions. These biopsies can significantly underestimate the mutational complexity of the full tumor complement present in the individual patient. Biopsies are often taken from a patient at a single time point to reveal a diagnosis. However, once a treatment is applied to the newly discovered genomic profile of the cancer, it exhibits a selective pressure on the tumor which allows resistant clones to survive and lead to eventual relapse and metastasis. In addition to the selection process by therapy, a biopsy may completely miss a clone on an opposing face of the same tumor mass, which could lead to relapse [19•]. Interestingly, many cancers shed cells, DNA, RNA, and proteins into the circulatory system. Theoretically, these shed cells and molecules may represent the patient's entire complement of tumor clones, which provides a unique tool for diagnosis. Assays of the blood, liquid biopsies, and blood-borne single-cell analyses (SCAs) are the next step to true precision medicine. SCA has previously shown that the clonal evolution of primary tumors is complex and branching in nature [23].

Due to the revolutionary development of cancer, a multifaceted treatment approach is crucial to form competent cancer therapy. It is obvious that many mutations and survival advantages must be present for these cancers to escape immune killing, eventually metastasize, and obtain plentiful supply of structural and energetic resources to continue growing. As these survival advantages accumulate over time, silent mutations also accumulate and these can provide the ability to outlive new selection pressures, e.g., chemotherapy resistance. In order to effectively treat such a multi-faceted disease, the administration of many diverse therapeutic interventions is required.

In the case of chronic myelogenous leukemia, the *bcr-abl* fusion gene, which results from a reciprocal chromosomal translocation, is likely the sole driver mutation that leads to cancer. Gleevec selectively targets the resulting fusion gene and halts its tyrosine kinase activity, resulting in significant tumor impact. Despite its effectiveness, the likelihood of relapse increases dramatically as time progresses [24, 25]. Many kinase inhibitors stop or slow growth by binding with their target in a specific location to result in inactivation, but cell death is not always associated. Thus, other cytotoxic drugs or immune activation against the tumor cells is probably required. These recent observations highlight the importance of a multi-targeted treatment regimen. Aside from limiting exposure to carcinogenic substances, possible treatment avenues include anti-angiogenesis drugs [26] and cytostatic drugs, effectively turning cancer into a chronic disease, which has been shown to prolong survival [27].

Since its inception in the 1970s, the Clonal Evolution Theory has been the subject of rigorous scientific analysis. In 2004, Matsui et al. described one hypothesis of clonal evolution in multiple myeloma [28•]. Specifically, their study highlighted the importance of "cancer stem cells" in producing phenotypically mature B cells. CD138, which is considered a specific plasma cell marker, is only expressed on terminally differentiated B cells [29]. This observation prompted Matsui et al. to investigate the nature of carcinogenesis in multiple myeloma. The researchers concluded that multiple myeloma was the result of cancer-causing stem cells [28•]. This study highlights the hypothesized branching of clonal evolution theory, which is key to treatment effectiveness.

As mentioned earlier, iatrogenic treatment can produce a Darwinian selection pressure on a developing cancer. This idea was further investigated in a study analyzing 682 newly diagnosed multiple myeloma patients [30•]. In several phase III trials, patients were treated with bortezomib, melphalan, and prednisone for 54 weeks unless severe toxicity was reported. Using a statistical modeling approach, the researchers identified trends within the treatment response data, aiming to improve trial design and understand the mechanisms of multiple myeloma development. It had been reported that bortezomib, a small-molecule proteasome inhibitor therapy, could induce complete tumor response and significantly increase survival [31, 32]. Following the expected clonal evolution theory outcome, patients began to relapse. Tang et al. developed a biologically logical mathematical design, which had been used to model progression in other diseases [30•], and tested their model by analyzing M-protein levels (which correlate with tumor burden in multiple myeloma [33]) to infer the validity of Clonal Evolution Theory. In their model-testing study, the researchers tested patient response data of 1469 patients and found that a hierarchical two-cell population mathematical model of multiple myeloma was most fitting. The proposed model suggested the existence of a multiple myeloma progenitor cell line with self-renewal capabilities and unique growth kinetics. In conclusion, the authors suggest

that progenitor cells are functionally distinct from other multiple myeloma cells in their own clonal population. Importantly, the study revealed that bortezomib therapy acts as a Darwinian selection pressure that can shape the outcome of the resultant disease phenotype [30•].

While multiple myeloma has shown clonal behavior, the theory has also been observed in chronic lymphocytic leukemia (CLL) when treated by a Bruton's tyrosine kinase (BTK) inhibitor [34•]. Ibrutinib is a small-molecule inhibitor that covalently bonds to Cys-481 in the ATP binding site of BTK [35]. Although ibrutinib has shown success in the clinic (71% overall response rate, 75% estimated progression free survival at 26 months), patients still progress after ibrutinib treatment [36]. These resistances have been attributed to developments of spontaneous mutations in the ibrutinib binding site of the *BTK* gene or the PLCγ2, a B cell receptor (BCR) signaling protein [37, 38]. Whole-exome and deep-targeted sequencing demonstrated *BTK-C481S* mutations and multiple *PLCG2* mutations in patients with CLL. Additionally, the authors uncovered driver mutations such as *EP300*, *MLL2*, and *EIF2A*, while one patient developed *trans*-differentiation into CD19-negative histiocytic sarcoma [34•]. Furthermore, the study revealed that ibrutinib-resistant clones were present before treatment initiation, passenger mutation, and demonstrated that ibrutinib therapy selected for these rare mutated and resistant cancer cells [34•]. Clonal Evolution has also been observed in solid tumors such as colorectal adenocarcinoma where studies have revealed two subtypes (TP53 and MAPK-PIK3CA mutants) that exhibited differing paths of mutational progression [39••]. The same study revealed that median progression-free survival for the MAPK-PIK3CA type was lower than control (8 vs. 13 months; univariate log-rank $p = 0.0380$, Cox model $p = 0.018$). The study concluded that differing patterns of genetic evolution in the tumor correlated with overall patient outcomes [39••].

In another study, mantle cell lymphoma (MCL) was shown to exhibit clonal behavior. Although the mechanisms of disease progression are unknown, past studies have uncovered that MCL primarily results from a t(11;14)(q13;q32) translocation that places the *CCND1* gene, encoding cyclin D1, next to the Ig heavy chain locus [40], which acts as a driver mutation for the disease. Analysis has shown that a common (occurring in 50% of patients with MCL) secondary event is a mutation or deletion of regions of the *ATM* gene, which regulates DNA damage response machinery [41]. Whole-exome sequencing of 27 biopsies from 13 MCL patients has shown 18 genes to be recurrently mutated. Some mutations have been observed before (*ATM*, *MEF2B*, and *MLL2*) but some were novel findings (*S1PR1* and *CARD11*) [42]. CARD11 is a scaffold protein that is important for BCR induction of the immuno-modulator NF-κB. Although only 5.5% of patients ($n = 173$) exhibited this genotype, in vitro studies have revealed that CARD11 mutants were resistant to the BCR inhibitor ibrutinib and NF-κB inhibitor lenalidomide [42]. Furthermore, the study revealed that these specific mutations were detectable in relapsed patient clones analyzed, supporting a branched evolutionary clonal process. Importantly, a point mutation in *MAP3K14* was identified in an MCL patient (MAP3K14 mutations are associated with ibrutinib resistance); this observation highlights the importance of complete tumor genome sequencing prior to therapeutic strategy development [42]. The authors conclude that MCL relapse is associated with de novo mutations in the resistant clones, which arise from a common progenitor. These data highlight the branching evolutionary behavior of cancer, which has been observed in other studies as well [43–46].

Immune Escape and the Evolving Role in Cancer Multi-Agent Therapeutics

The human immune system is equipped to kill human and non-human cells, as well as non-cellular (parasite) invaders. Those immune cells that recognize normal human host tissues are eliminated or suppressed by other immune regulatory systems [6•]. As suggested above, the emergence of initial cancerous cells is believed to be met by the active immune system and either eliminated or held in equilibrium by these normal functioning immune cells [8•]. Only by a cancer cell developing a survival trait through mutation, or other mechanisms (described later) can this immune cell recognition and control be overcome. The ability to avoid immune killing by cancer cells appears to be a critical step in the malignant process and is termed "immune escape" [8•].

The normal immune system has checkpoints that instruct immune cells to reduce their activity in a time frame where bacteria or viruses have been eliminated, 7–10 days or so [47]. These checkpoints are found in increasing levels on activated immune cells, but once critical mass is reached the activated state is reversed. This normal control system is essential to turn off immune reactions when they are no longer required, e.g., eliminated/controlled viral infections.

Cancers cells, through mutations and protein induction, can develop, express, and mimic these signaling checkpoints on their surface or in their environment much like camouflage can make military targets look like normal elements of the landscape [48•]. In essence, these cancer cell-associated mutational abnormalities disguise the true identity of the cell by signaling to immune cells to suppress a proposed kill mechanism, halting its cytotoxic response. The revolutionary expression therefore protects cancer cells. For example, breakpoint cluster rearrangements in chromosome 9p lead to elevated expression levels of PD-L1 [48•], permitting the clone to "escape" the immune system's wrath while providing an essential survival advantage.

More than 18 checkpoint processes have been identified that regulate immune cell activity. [47] Therapies targeting CTLA-4 and PD-1 on immune cells were the first to be developed. The goal of these therapies is to inhibit the checkpoint molecules from interacting, thereby blocking the immune cell's normal suppression mechanisms and increasing its survival and thus its activity. In principle, this can be thought of as removing the brake of specific immune cells (see Table 1 for approved products and indications).

The PD-1:PD-L1 interaction is a paragon of molecular checkpoint inhibitors of the normal immune system. The gene and protein PD-1 were first discovered in 1992 [49]. Furthermore, expression of PD-1 is known to be induced after activation of T cells [6•, 50]. The ligands of PD-1, PD-L1, and PD-L2 are found on a variety of hematopoietic and non-hematopoietic cells [51–53]. The interaction between PD-1 and its ligands leads to inhibition the PI3K pathway, decreasing T cell receptor signaling and survivability of the cytotoxic T cells [54]. Thus, when functioning normally, the expanded cytotoxic population is inactivated and decreased back to memory cell baseline numbers by apoptosis after an infection has been controlled. The system is in place for self-protection and to prevent autoimmunity. In fact, mutations in the PD-1 and PD-L1 gene systems have been associated with autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis [55, 56]. However, tumors can take advantage of this system to bypass detection and destruction by displaying PD-L1 on their surface [48•]. Cancers, including melanoma, lung, breast, bladder, renal cell, pancreatic, myeloma, and lymphomas, have been shown to express PD-L1 and interact with PD-1 on activated T cells to inhibit their function [57–66].

Blockage of PD-1, with monoclonal antibodies for example, structurally inhibits binding to PD-L1, which maintains immune activity and allows for tumor cell recognition and control [67]. Inhibition of PD-L1 also interferes with the PD1:PD-L1 interaction and has been shown to block T cell apoptosis while sustaining immune activity [68, 69]. Overall, these novel checkpoint inhibitors have been shown to benefit survival even in patients with advanced disease and previous treatment. Deeper investigations into the PD-1:PD-L1 checkpoint, as well as other characterized pathways, are necessary to achieve the truly multi-faceted approach that modern cancer treatment requires.

All the immune system checkpoint inhibitors work to control the immune reaction. These normal control mechanisms, however, can vary in effectiveness between different tissues and states of the same tissue, e.g., the local environment [46]. PD-1 is not expressed on the surface of naïve T cells, but upon activation, expression is turned on within the cytotoxic T cells [6•]. PD-1 mostly downregulates inflammatory reactions in the periphery, limiting autoimmunity [70, 71]. This is why anti-PD-1 monoclonal antibody therapies are associated with less aggressive autoimmune adverse events than some other checkpoint inhibitor therapies such as anti-CTLA-4 antibodies. The interaction of CTLA-4 and B7–1 is known to induce IDO expression leading to suppression of T cell response [72, 73]. In vivo studies have shown that inhibition of IDO with 1-methyl-tryptophan can result in T cell responses against tumors [74–76].

With blockade of the PD-1:PD-L1 interaction, the AKT pathway is not activated and the downregulation of normal immune activity and elimination of the activated T cells through apoptosis does not occur. The intervention leaves previously activated T cells in their current state and permits longer survival. By increasing the lifetime of activated T cells, anti-PD-1 and anti-PD-L1 therapies have achieved significant responses and long-term survivals, leading to their worldwide approvals for multiple indications (see Table 1). In the thymus, PD-L1 is also expressed on the thymic cortex, on thymocytes, and in the thymic medulla and is important in both positive and negative T cell selection [77].

Although checkpoint inhibitors can stop the normal T cell reaction regulatory process, interestingly, T_{reg} cells also play an important role in controlling immune reactions. The immune system controls are divided between checkpoints and cellular controls such as T_{reg} cell activity. Checkpoints such as PD-L1 can cause T_{reg} cell-specific population numbers and immune regulatory activities to increase [78]. Some evidence suggests that, in a normally functioning immune system, checkpoints binding to a ligand (PD-1:PD:L1) not only downregulate $T_{cytotoxic}$ cell activity but also increase T_{reg} cell numbers and activity, causing further inhibition of immune reactions. These studies have found that this phenomenon is signaled through the AKT/mTOR and PTEN pathways [78–80]. While T_{reg} cell activity is important, NK cells are also capable of killing normal human cells as well as cancer cells. There is now evidence suggesting that certain NK cells in tumor bearing mice can be primed to express several checkpoint ligands such as PD-L1, CTLA4, and LAG3, which bind to activated dendritic cells to additionally downregulate the immune response [81•, 82].

Despite its seemingly simple construction, the PD-1:PD-L1 checkpoint is actually much more convoluted (see Fig. 1). Tumor-infiltrating lymphocytes are often regarded as a sign of immune activation in tumor samples [83], although their presence can also be a negative predictor [84]. Recent data demonstrates that inflammation and inflammatory cytokine expression by T cells (interferon-γ specifically) can induce normal and tumor tissues to express PD-L1 [85, 86]. These human tissue investigations strongly suggest that an additional downregulation system occurs in normal tissue expression of PD-L1 (B7-h1). Furthermore, the immune system's action against tumor cells may, in specific circumstances, drive the expression of checkpoint inhibitors, thus slowing and even stopping the immune response.

An additional mechanism of immune response suppression comes from the innate branch of the immune system. This cellular system is also complicated but has begun to be elucidated [87•] These myeloid-derived suppressor cells (MDSCs) are phenotypic of either neutrophils or monocytes/macrophages, but have different immune functions based on their location and environment. These cells can impact T cell tumor tolerance, dendritic cell inhibition in draining lymph nodes, and the reactions against the tumor itself. Aberrant chemokine production by tumors attracts MDSCs to the tumors, as shown by the abundance of MDSCs in many cancers. Additionally, MDSCs have been shown to increase PD-L1 expression in tumors [87•]. MDSCs have different functional activities near the tumor, in the lymph nodes, spleen, and other organs. In the future, therapeutics will need to target many, if not all, of the location-dependent suppression mechanisms of the MDSCs to stop their pro-cancer activities.

The data suggest that there is a balance between active killing action of T and NK cells and immune downregulators (checkpoints, T_{reg} cells, MDSCs) that determine the progression of disease or lack thereof.

These factors make the following therapeutic strategy important to consider (see Fig. 1):

1. Multiple agents (one to eight or more interventions) to inhibit mutated cancer genes providing survival systems

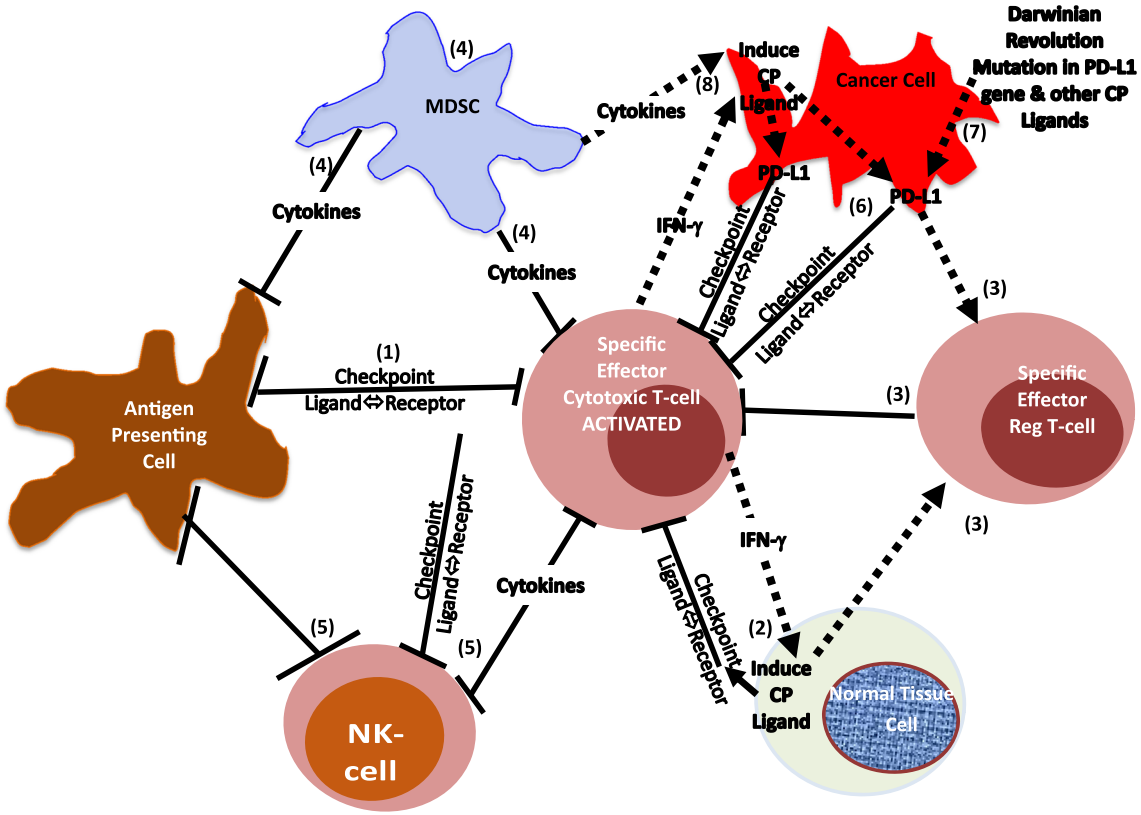

Fig. 1 Interactions of cancer clones, normal tissues/cells, and normal human checkpoint systems. Both molecular and cellular interactions can significantly influence the normal immune system. Suppression and activation signals are represented as *solid blunted arrows* and *dotted arrows*, respectively. The local normal recognition and killing immune system cells are suppressed by a cascade of molecular and cellular systems including normal surrounding tissues cells that have been induced to express checkpoint ligands, normal MDSCs, normal APCs, and T_{reg} cells. Additionally, the cancer cell can be induced to express checkpoint ligands, mutate to express checkpoint ligands, or both to suppress the T cells and NK (natural killer) cells. (1) The activated T cell expresses checkpoint (CP) molecules, e.g., PD-1. These molecules bind to their ligand, PD-L1, or PD-L2 on APCs or dendritic cells and suppress their activity, thus suppressing activation of more T cells. (2) Cytokines elaborated by the normal immune (innate and adaptive) inflammation process induces normal local host cells to express checkpoint (CP) ligands that can in turn suppress the immune reaction. (3) Checkpoint inhibitor expression is recognized by T_{reg} cells and causes expansion of T_{reg} cell populations that can suppress the T cell reaction process. (4) Myeloid-derived suppressor cells are attracted to the inflammatory environment. MDSCs express cytokines that can directly suppress the T cell recognition and killing and downregulate APCs. (5) NK cells are also believed to be downregulated in this inflammatory milieu of cytokines, cellular elements, and suppressive molecules. They then can downregulate the activated and expanded APC back to baseline. (6) The T cell expression of cytokines can induce malignant cells to express checkpoint ligands, directly inhibiting T cell recognition, binding, and killing. (7) The Darwinian revolution can result in mutations within the malignant cell that cause checkpoint ligand expression, or other immune-suppressing molecules that can directly cause suppression of the immune reaction process. (8) The immune reaction cells can directly cause (like in normal tissues) upregulation and expression of checkpoint ligands that suppress themselves

(driver mutations like growth/apoptosis pathways, cellular components needed for daughter cell formation, and other nutrients for enzymatic and growth energy supply)
2. Block checkpoint inhibitors to allow continued activity of T cells (one to five or more interventions)
3. Increase the number of natural specific anti-tumor immune cells within the tumor environment, still under investigation (this is the premise behind anti-cancer vaccines plus checkpoint inhibitors) (one to five or more interventions)
4. Augment anti-cancer cellular destruction by targeted cellular therapies, e.g., CAR-T cells (one to three or more interventions)
5. Additional therapeutics to stimulate or maintain activity of dendritic cells, innate cells, and adaptive cells (one to four or more interventions)
6. Downregulate cell-mediated suppressors of immunity, e.g., anti-T_{reg} cells, MDSCs, and other therapies (potentially one to five or more interventions)
7. Again, block immune associated expression of checkpoint ligands that may be upregulated by active immune responses in the tumor region, e.g., IFN-γ direct stimulation of checkpoint ligand expression by normal and cancer tissues (potentially one to three or more interventions).

Due to the complexity of these various intertwined immune regulating systems, multi-agent therapeutic attacks may be necessary to alter the balance of activated immune cells that kill cancer and exhausted immune cells that no longer recognize and/or kill cancer cells.

Conclusions

Malignant cell transformation, constant mutations, and uncontrolled growth resulting in the cancer patient's death have perhaps been the most frustrating and scientifically complicated diseases medicine has attempted to treat. Cancer begins slowly and often grows slowly. The immune system can usually recognize these early cancers and eliminate them or keep their growth in equilibrium. However, these malignant cells often develop (or may already have) a mechanism to generate mutations within the genome at an alarming rate that we have described as Darwinian revolution [6•]. Most of these mutations are detrimental to the cancer cell, leading to its own death. Other mutations, however, are silent in their effects or provide a survival benefit. These mutations lead to the unchecked, continuous cell growth, even though the actual kinetics may not change. Since the continuous growth requires more nutrients than normal cells, mutations that cause faster nutrient acquisition and energy availability are common survival benefits observed in cancer tissues.

Importantly, cancers are often still recognized as different and capable of being killed by the host immune system. One major transformation from the equilibrium state is the ability to escape detection, recognition, and subsequent killing by the immune system. Mutations appear to be able to confer immune escape by expressing cloaking proteins that camouflage the identity of the cancer cell. These disguises can allow the cancer to avoid immune detection or to turn off immune cytotoxic pathways. However, immune control systems usually function normally in many aspects and downregulate immune reaction before the cancer cells are completely destroyed. This phenomenon is often referred to as T cell exhaustion. In fact, it is a combination of checkpoint systems downregulating T, NK, and dendritic cell activity while upregulating cellular immune control systems like T_{reg} cells and myeloid-derived suppressor cell activity. Furthermore, inflammatory reactions and tumor cell elaboration of certain cytokines can also upregulate normal and cancer tissue to attract suppressive normal cellular systems and express checkpoint downregulators to further suppress the immune system (see Fig. 1).

In final accounts, cancer cells will develop tens to hundreds of mutations that provide a potential for several survival benefits. In the normal function of the immune system, reduction of activity allows tumors to develop additional immune suppression capabilities. Both arms of the immune system are the only defenses against cancer cells by the human host. As the cancer cells grow and develop over time, more survival traits accumulate as mutations are acquired. Some of these mutations provide suppression signals to the host normal immune system. These normal host innate and adaptive immune cells are the only non-pharmacological elements available to stop the cancer cells' growth and able to eliminate these malignant cells from the patient. Camouflaging mutations are a major survival mechanism of cancers. Additionally, the normal immune control mechanisms applied to the cancer environment can induce molecular and cellular downregulation processes (see Fig. 1).

Therapeutic strategies must consider the (1) multiple mutations and (2) the induced survival traits that influence the cancer cells. These appear to require therapeutics that stop cancer growth, initiate apoptosis, and eliminate structural plus energy sources. However, in addition to direct malignant clone therapy, combination therapeutic strategies must include the control of normal and abnormal immune processes allowing recognition and elimination of these cancer cells.

This strategy to eliminate cancer cells will require multiple parallel and sequential therapies attacking various clones of cancer with differing complements of survival traits, while releasing specific immune cells to clear tumor cells that can sustain growth. We believe that cure is possible in combining many (7–30 or more) therapeutic measures that target this multitude of abnormalities that lead to malignancy-associated human death.

Compliance with Ethical Standards

Conflict of Interest Jonathan L. Messerschmidt, Prianka Bhattacharya, and Gerald L. Messerschmidt declare they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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