

Chapter 2

The Role of the Immune System and Immunoregulatory Mechanisms Relevant to Melanoma

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Abstract A hallmark of melanoma is its inherent immunogenicity through both innate and adaptive immune mechanisms. However, a number of factors inhibit these immune responses through intrinsic mechanisms in tumor cells or adaptive resistance triggered by the immune response via negative feedback. Understanding how these processes are balanced in context of pathways regulating T-cell activation, migration, and differentiation, and T-cell dysfunction in tumors has become a critical area of research. This chapter describes the immunoregulatory mechanisms in the melanoma tumor microenvironment and how positive and negative signaling elements can be harnessed to facilitate enhanced anti-tumor immune responses through immunotherapy.

Keywords Melanoma • Immune cells • Immunotherapy • Anti-tumor immunity • Cytokines

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2.1 Introduction

One of the key starting points in a discussion of anti-melanoma immunity is whether the immune system evolved to recognize tumor antigens and mount an adaptive (T-cell-mediated) antitumor immune response together with key components of innate immunity operating either in a facilitative or inhibitory role. This fundamental question needs to frame any detailed discussion of the mechanisms of antitumor immunity and the development of cancer immunotherapies relevant to melanoma. The adaptive immune system has a remarkable capacity to recognize foreign pathogens, such as bacteria, viruses, and fungal infections as one of the most primordial life-preserving functions throughout evolution. This powerful response against foreign invaders however is also counterbalanced by equally powerful immunoregulatory mechanisms designed to limit damage to normal tissues (autoimmunity) and prevent runaway immune responses (immunopathology) that can ultimately kill the host. Both of these fundamental immunological processes also intersect during wound healing due to the need to curtail any opportunistic infections in wounds or due to tissue damage that may be caused by infection while at the same time limiting inflammation in resolving parts of the wound to initiate tissue repair. From an evolutionary biology perspective, both these two contrasting functions of the immune system evolved over millions of years culminating in mammals and humans as a mechanism of protection against infectious disease while preventing immunopathology mainly as a protective mechanism during reproductive years, thus perpetuating the species. In addition, these processes evolved to their present forms in context with the development of an adaptive immune system consisting of highly specific antigen receptor-expressing lymphocytes [T-cell receptor (TCR)-expressing T cells mediating cell-mediated immunity and immunoglobulin receptor-expressing B cells mediating antibody-dependent immunity through opsonization] together with a more primordial innate immune system that developed cross-circuiting or cross-communication with adaptive immunity as a critical initiator of T- and B-cell responses as well as suppressive lymphocyte responses.

Thus, with these biological realities in mind, a fundamental question that emerges is whether the immune system also “evolved” to fight cancer (abnormal growth and differentiation of self-tissues that threaten life) as an intrinsic process during evolution to preserve the species or is the anticancer immune response an evolutionary “fluke of nature” based on how the immune system evolved to distinguish self from non-self (foreign antigens). Cancer can be viewed as an “altered non-self” that the immune system by default evolved some capacity to recognize, but rather inefficiently due to the suppressive counterbalances (e.g., central T-cell tolerance during T-cell differentiation in the thymus gland, immunosuppressive cytokines such as TGF- β , Foxp3⁺ T-regulatory cells, myeloid cells during wound healing) that exist that protect us from autoimmunity and immunopathology. Aging is another parameter that now enters this discussion. In this context, cancer (including melanoma) is really a disease of aging where cumulative genetic and tissue damage (e.g., from cumulative UV radiation) together with chronic inflammation leads to abnormal cellular proliferation, immortalization, invasiveness, and then ultimately metastatic

spread that kills the host. As human aging has become a reality with lifespan increasing beyond original evolutionary constraints, the immune system is now faced with a new and unexpected selective pressure to somehow modify its essential evolutionary function away from simply fighting infection to maintain life only through the reproductive years. However, both the innate and adaptive arms of the immune system significantly weaken during aging, as manifested in thymic involution reducing naive T cells, reduced NK cell function, suppressed dendritic cell activation, chronic inflammation mediated by abnormally activated macrophages, and oligoclonal expansions of T-cell clones (e.g., CMV-specific CD8⁺ T cells) together with senescence (caused by telomere shortening and terminal T-cell differentiation). Ultimately, all these evolutionary processes and consequences are shaping how the immune system is “learning” to cope with melanoma and other forms of cancer.

Due to the damage to local tissues caused by primary tumors and metastatic growth, all tumors, including melanoma, are intrinsically “immunogenic” and drive a wound healing response attracting innate myeloid and granulocytic cells. The inflammation and damage to both normal stromal tissue and cancer cells in this process releases tumor antigens presented on class I and class II major histocompatibility complex (MHC) molecules activating CD8⁺ and CD4⁺ αβ TCR⁺ T cells. Tumors can induce an immune response both systemically and at the tumor site against tumor antigens inducing T-cell responses and other stress-induced molecules that are recognized by innate lymphocytes (NK cells and γδ T cells) and antigen-presenting cells (dendritic cells). However, unlike a successfully resolved infection by a foreign pathogen or a normal wound healing response, it has become readily apparent that immunosuppressive mechanisms both systemically and in the tumor microenvironment are triggered during cancer progression and advanced cancer (manifested as metastases from seeded circulating tumor cells), limiting the effectiveness of an antitumor immune response. However, despite these processes, *de novo* adaptive immune responses against both primary and metastatic melanoma occur in almost all patients and, thus, questions arise as to what role these responses play in the overall course of the disease and patient survival, why some patients with melanoma mount more effective adaptive immune responses against both local and disseminated tumors manifested by higher lymphocyte infiltration into the tumor bed that can be boosted by immunotherapy, and what biomarkers are associated with this enhanced level of “immunocompetence.”

It is in this conceptual framework that we will discuss the key processes regulating immune responses against melanoma both at the tumor site and systemically, the cellular components of the immune infiltrates in the melanoma tumor microenvironment, and how tumors can regulate immune responses in the host systemically in uninvolved secondary lymphatic tissues (tumor-draining lymph nodes and uninvolved nodes, as well as the spleen). We will introduce the concept of how and why melanoma is considered an “immunogenic” tumor regulated by inflammation and how critical inflammatory molecules can either be drivers of cancer cell survival and growth or act as initiators of adaptive cytotoxic T-cell responses that can eradicate tumor cells by recognition of tumors antigens presented on MHC molecules. In some cases, tumor-associated stromal cells, such as tumor-associated fibroblasts (TAFs) and endothelial cells (from tumor angiogenic processes), can also be targeted. We

will also discuss how the normal checks and balances that evolved in the immune system during evolution to prevent runaway inflammation and autoimmunity, manifested as negative feedback mechanisms at different stages of the immune responses or during normal T-cell differentiation, can inhibit adaptive immune responses against these “wounds” and some of the key molecules and signaling pathways involved in this process such as the T-cell “checkpoint” molecules (e.g., CTLA-4 and PD-1) that have emerged to be exciting new cancer immunotherapy targets. Lastly, we attempt to bring together the concepts discussed into a unifying set of principles that can be used to drive immune biomarker research and how these immune system biomarkers can be used as prognostic tools or as tools to determine which patients are more apt to respond to immune intervention (immunotherapy) against their melanoma, especially at the advanced stages. Our hope is that these biomarkers can be eventually used to select patients for specific types of immunotherapies or combination therapies with targeted drugs or chemotherapies. A minimal knowledge of basic immunology is assumed, including the major components of the innate and adaptive immune response and knowledge of T-cell antigen recognition and activation processes involving positive costimulatory molecules and negative costimulatory molecules or checkpoints, the major types of T-cell responses, as well as the major cytokines involved. There are some excellent textbooks and overviews on basic immunology that the reader can consult if needed [1, 2].

2.2 Melanoma as an “Immunogenic” Tumor: Lymphocytes and the Tumor Microenvironment

2.2.1 Melanoma Is a Heterogeneous Entity of Tumor Cells, Hematopoietic Cells, and Resident and Nonresident Stromal Cells Regulating the Melanoma “Wound”

Melanoma is a heterogeneous entity of cells comprised of distinct clones of cancer cells, with different proliferative and invasiveness capacities, and stromal cells. In the tumor microenvironment, constant interaction between cancer cells and different stromal cells could either promote tumor rejection or tumor progression. In many cases, melanoma cells appear to “manipulate” the stromal cells within or surrounding the tumor to drive their growth, survival, and evasion from attack by immune cells. The stromal compartment in the tumor microenvironment itself is complex and comprised of numerous cell types that are categorized as resident and nonresident stromal cells [3]. Resident stromal cells including tumor-associated fibroblasts (TAFs), tumor-associated endothelial cells (TECs), and mesenchymal cells are cell populations that reside within stroma. Melanoma cells and resident stromal cells are a good source of a broad array of growth factors, proinflammatory cytokines (IL-6, IL-8, TNF- α) [4, 5], and chemokines (CXCL12, CCL5) [6–8]. Acting in either an autocrine or paracrine manner, tumor-derived cytokines and

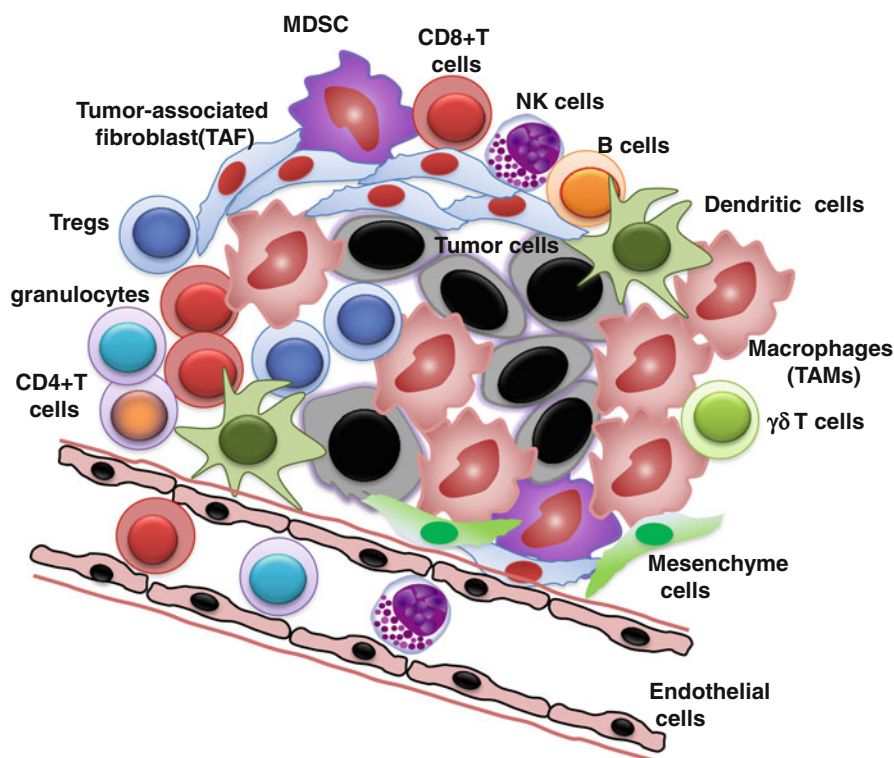


Fig. 2.1 Tumor microenvironment in melanoma. The tumor microenvironment is composed of many cell types that are recruited through the production of cytokines and chemokines. These include nonresident stromal cells such as tumor-associated fibroblasts (TAFs) and mesenchymal cells as well as various innate and adaptive immune cells [dendritic cells, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSC), CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, granulocytes, B cells, and NK cells]. Immune cells can be found within the tumor (intratumoral) as well as surrounding the tumor (peritumoral)

chemokines support tumor growth while recruiting nonresident stromal cells, comprised of various innate and adaptive immune cells (such as dendritic cells, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), T cells, $\gamma\delta$ T cells, granulocytes, B cells, and NK cells) to the tumor site (Fig. 2.1).

In many melanoma tumors, lymphocytes, especially CD8⁺ T cells that recognize melanoma antigens (such as Melan-A/MART-1, gp100) and NK cells, can be found to differing degrees [9, 10]. These cells can produce high amounts of IFN- γ and express high levels of cytotoxic molecules such as granzyme B and perforin and play a positive role in limiting rising malignancies and in facilitating tumor rejection. The presence of CD8⁺ T cells has been correlated with better clinical response and survival in patients with melanoma and with other cancer types [11–13]. The infiltration of other cell types, such as IFN- γ -secreting- $\gamma\delta$ T cells and NK/T cells,

within the tumor tissue has also shown a favorable clinical outcome [14, 15]. However, in parallel to the infiltration of antitumor immune cells, negative immune regulators with immunosuppressive function such as TAMs, suppressive T-regulatory cells (Tregs), and MDSCs also accumulated in melanoma tumors [16–19]. These cells contribute to cancer progression by limiting the efficacy of CD8- and NK cell-mediated antitumor responses via various immunosuppressive mechanisms such as secretion of TGF- β , IL-10, IL-6, and IL-1, IDO expression in the tumor microenvironment reducing tryptophan, sequestration of growth factors such as IL-2 from T cells, and the release of factors mediating oxidative stress [20–22]. In this regard, the accumulation of Tregs in the tumor microenvironment significantly contributes to a decline in antitumor CD8⁺ and Th1 CD4⁺ T-cell responses and decreased overall survival and relapse-free survival [23, 24]. In addition, the presence of immunosuppressive TAMs exhibiting M2 phenotype was associated with poor survival in patients with melanoma [16, 17].

Furthermore, close interactions between positive and negative immune regulators with cancer cells in the tumor microenvironment could result in the polarization of “good” immune cells to “bad” immune cells that turn from mediating “antitumor” to “pro-tumor” effects. For instance, the presence of the immunosuppressive cytokine TGF- β , together with a low antigen signal in the tumor microenvironment, facilitates the conversion of conventional CD4⁺ T cells to induced regulatory T cells (iTregs) that can suppress antitumor responses mediated by CD8⁺ cytotoxic T cells and NK cells. In addition, many of the infiltrated-CD8⁺ T cells in melanoma tumors were found to be dysfunctional [25–27] or incompletely differentiated in a TGF- β -dependent manner [28], thereby lacking the capability to mediate tumor rejection. Thus, the diverse communication of immune cells with cancer cells and within the tumor microenvironment dictates the final outcome of anti- or pro-tumor responses.

Moreover, the extent of infiltration by various antitumor and immunosuppressive immune cells, together with pro- and anti-inflammatory cytokines and chemokines that are present in the tumor microenvironment, contributes to different inflammation states of tumors: non-inflamed, over-inflamed, and well-balanced inflammation. The overall inflammatory nature of a tumor, either being non-inflamed, over-inflamed, or having well-balanced inflammation, would rely on how cancer cells and stromal cells interact and on their activation state. In a well-balanced inflammation tumor, both positive and negative immune regulators are present in the tumor microenvironment and operating to promote “good inflammation” that could lead to tumor rejection. However, some melanoma tumors are non-inflamed and lack T-cell accumulation in the tumor microenvironment, whereas some tumors are over-inflamed and contain massive accumulation of innate immune cells that promote a “bad” inflammation state and facilitate tumorigenesis [9]. This concept will also be discussed in context of tumor-related biomarkers later. Recently, specific gene expression patterns that were associated with different states of inflammation and immune cell infiltration of melanoma tumors have been identified. In this regard, CD8⁺ T-cell-infiltrated but not non-inflamed tumors have showed a type I IFN transcriptional profile as well as the expression of key chemokines, and these gene signatures may implicate an association with the activation of innate immune

responses [29, 30]. Melanoma patients having T-cell-inflamed tumors showed expression of a chemokine signature for T-cell recruitment (CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10) had a more favorable clinical outcome [9, 30].

Paradoxically, the activation of antitumor-related cytokine secretion, for example, IFN- γ , which is traditionally considered to be important in mediating tumor-specific responses, has been shown to participate in immunosuppressive mechanisms. For example, the presence of Th1 producing IFN- γ cells was linked to the promotion of MDSC accumulation and the lack of efficient antitumor responses [31]. Recently, Spranger et al. have showed that infiltrating-CD8 $^+$ T cells were responsible for the recruitment of Tregs and for an increase of IDO and PD-L1 expression in the melanoma tumor microenvironment [20]. These data suggest that strong inflammation mediated by IFN- γ could activate a negative feedback immune response by recruiting immunosuppressors (e.g., Tregs and MDSC) to counteract the local inflammation and to suppress the tumor-specific responses. Taken together, it is crucial to understand how the interaction between stromal cells and cancer cells contributes to the different state of inflammation and outcome of immune responses to further improve current immunotherapy against melanoma.

2.2.2 The Concept of Melanoma Tumor Antigens Recognized by T Lymphocytes of the Adaptive Immune System

Just like the majority of cells that compose the human body, melanoma tumor cells express class I major MHC molecules. Although the expression level may vary from tumor to tumor, these MHC class I molecules are a way for the immune system to identify a tumor cell and engender its destruction. Each MHC class I molecule expressed at the cell surface forms a complex with an 8–12-amino acid long peptide. Peptides are usually the degradation product of an intracellular protein or from the extracellular compartment if the protein was internalized by a professional antigen-presenting cell (APC) like dendritic cells (DCs) or macrophages (Fig. 2.2). The complex formed by the MHC class I molecule and the peptide at the surface of the cell can be recognized specifically by the TCR of a CD8 $^+$ T lymphocyte (CD8 $^+$ T cell) and trigger different activation signals leading to proliferation and/or cytotoxic functions. A similar process exists for CD4 $^+$ T-helper cells, but in this case, longer 10–15-amino acid length peptides (usually derived from extracellular and secreted proteins) are presented on MHC class II molecules.

During development, CD8 $^+$ T cells have been selected in the thymus to recognize MHC class I in a process called positive selection. A second step called negative selection aimed to eliminate T cells recognizing self-peptide to prevent autoimmune responses. Mature CD8 $^+$ T cells spared from that “negative selection” are believed to be able to eliminate only cells presenting peptides from foreign proteins such as a virus. A protein from which a derived peptide triggers the recognition and action of a T cell is called an antigen. This definition extends further than viral, bacterial, fungal, and other “foreign” proteins to also overexpressed self-proteins or antigens

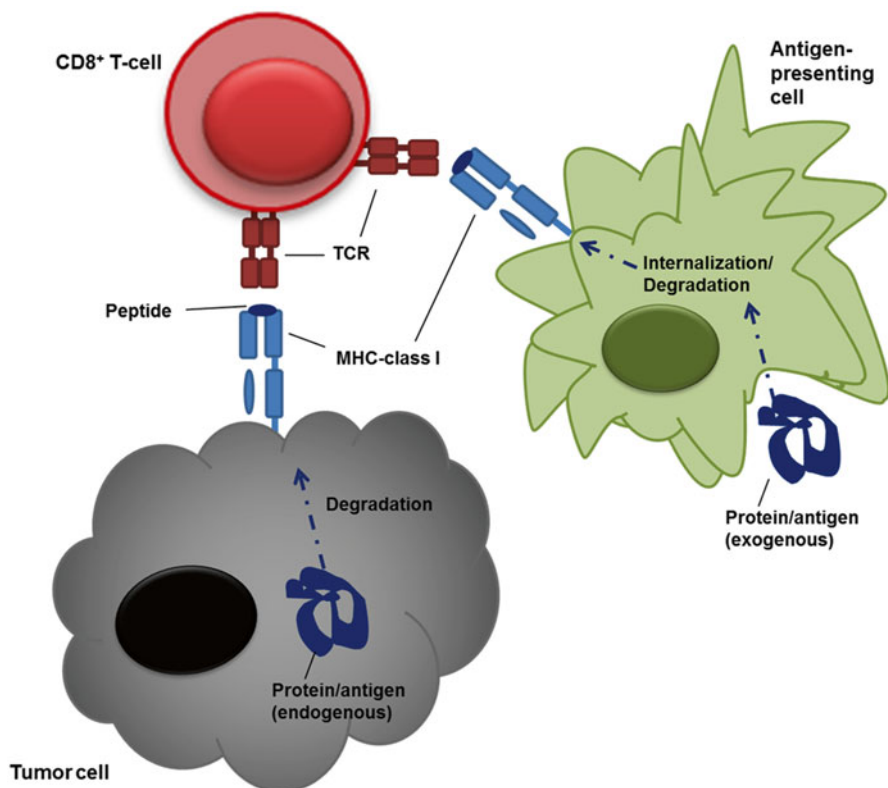


Fig. 2.2 MHC class I peptide presentation. The T-cell receptor (TCR) of a CD8⁺ T lymphocyte (CD8⁺ T cell) can specifically recognize peptides presented at the cell surface as a complex with MHC class I molecules. These peptides are the result of protein degradation. Intracellular proteins, for example, in tumor cells, can be degraded into small peptides which, if they possess strong affinity to bound the intracellular MHC-I molecule, will then migrate to the surface of the cell to be presented to a CD8⁺ T cell. If MHC-I-peptide complex is recognized by the T cell, this will trigger different activation signals leading to proliferation and/or cytotoxic functions of the T cell. Peptides can also originate from degradation of extracellular proteins. These proteins are internalized by a professional antigen-presenting cell (APC) like dendritic cells (DCs) or macrophages before being degraded and loaded on MHC-I to be presented, as a complex, to the CD8⁺ T cell

induced under conditions of stress and cancer, as we will further demonstrate in this section. These overexpressed self-proteins normally ignored by the immune system, together with protein products from mutated genes, constitute what are termed tumor-associated antigens (TAAs).

TAAs are generally defined as proteins expressed by tumor cells with a weak or nonexistent expression in normal tissues, especially vital tissues such as the brain, lungs, and heart. TAA immunogenicity, which is defined as its capacity to be presented on MHC (i.e., a peptide-bound MHC class I or class II molecules on the cell surface) and recognized by the immune system (i.e., peptide-MHC complex recognized by a TCR), has to be proven before being designated as a TAA. The field

has actually attributed four qualities that would constitute the ideal TAA [32]. In addition from being absent from normal tissues, it is preferable that TAA are involved in tumor progression so it is more beneficial when targeted. The ideal TAA also has known peptides that can be recognized by T cells and presented naturally, complexed with MHC class I and class II at the surface of tumor cells [32].

The TAA can be classified into four families: shared cancer/testis antigens, differentiation antigens, antigens overexpressed in cancer cells, and antigens derived from somatic mutations of tumor cells [33]. It is in the cancer/testis family that we find the first TAA to have been identified, MAGE-1 [34]. These TAA were expressed during development but are now silenced in mature, normal cells with the exception of spermatozooids, which makes these antigens great targets with very few off-target effects (side effects) for the patient. However, unsuspected low levels of expression of these proteins in normal indispensable tissues can lead to high toxicity like it was observed when the TAA MAGE-A3 was targeted with an engineered MAGE-A3-specific T cell, resulting in neurologic problems and even death [35]. NY-ESO-1, which is found in melanoma tumor cells as well as other cancers, is part of this family [36]. The TAAs in the melanocyte differentiation family are often expressed at lower levels in normal cells from which the tumor has arisen. The most common TAAs in this family are the proteins gp100, tyrosinase, tyrosinase-related protein 1 and 2 (TRP1 and TRP2), and MART-1, which are expressed in melanocytes through their role in melanin biosynthesis but also found highly expressed in melanoma cancer cells [37]. Targeting these TAA in immunotherapy can usually give rise to non-life-threatening side effects like skin depigmentation. The difference between the overexpressed antigen family and the differentiation antigens is that the expression of the overexpressed antigens is not restricted to the tissue where the tumor emerged but is also found in other normal tissues [33].

The last category, the mutated Ag, has been a subject of great interest lately with the use of exomic sequencing methods to rapidly identify mutations generating mutated proteins/peptides that can be recognized by T cells that have infiltrated the tumor [38, 39]. It was recently demonstrated using a murine model of chemically induced tumors that some protein mutations generated highly immunogenic peptides sufficient to mediate the tumor eradication by T cells recognizing the specific mutation [38]. This represents a major advantage when targeting TAA for therapy given the absence of expression of this type of mutation in normal tissues. The T cells that are specific against this mutated protein either do not recognize or have a weak recognition for the wild-type version of the protein or the wild-type version is simply not expressed. Conversely, when some of the resident tumor cells lack expression of such an immunogenic peptide, the cancer immunoediting phenomenon occurs, which will be subsequently discussed, and gives the tumor cells the capacity to overpower the action of the immune system [38]. In humans, it is a well-known fact that cancers caused by mutations such as in smoke (MCA) for lung cancer or UV for melanoma are themselves enriched with mutations. It was recently demonstrated that a mutated antigen isolated from a melanoma tumor cell line that was recognized by autologous tumor-infiltrating lymphocytes (TILs) was responsible for tumor regression when these TILs were massively expanded *in vitro* and adoptively transferred to the patient (discussed in a later section) [39].

There is another category of TAA which the literature does not include in the four families: the viral TAA. Some solid tumors like nasopharyngeal cancer originates from a viral infection which gives rise to immunogenic peptides coming from that virus and also expressed by tumor cells [40]. For melanoma, viruses are not considered a primary cause of tumor genesis; however sequences of the K-type human endogenous retrovirus (HERV-K) were found in melanoma tumor cells but not in normal melanoma [41]. UV radiation has been reported to induce HERV-K activation which could in turn participate in the malignant transformation of melanoma cells [42, 43]. A peptide from the HERV-K-MEL found in melanoma was able to bind MHC class I and to be recognized by CD8⁺ T cells taken from the blood of melanoma patients [44]. Even though HERV-K-MEL was not initially classified as a TAA in the four families of TAA because of its endogenous genomic origin, the fact that it is expressed in cancer cells and not normal cells, and that it is immunogenic and may participate in driving tumor progression, qualifies it as a TAA for melanoma.

2.3 The Immune System Can Be “Two-Faced” Either Inhibiting or Facilitating Early Melanoma Progression

The immune system can be a “two-edged sword” in regulating tumor development or tumor control. Research is now just beginning to understand the factors that play a role in the immune system helping drive cancer versus those playing a role later in eradicating cancer cells through innate and adaptive mechanisms both at the earliest stages of cancer initiation (called “tumor immunosurveillance”) or at more advanced stages through immunotherapeutic interventions. The line between these two processes is still “fuzzy” and is still being defined. Moreover, the immune system can be a driver of cancer in two scenarios: (1) early cancer formation and (2) generating immune-resistant tumor cell subsets in more advanced disease through immunoselecting antigen-loss variants, variants that lose key ligands (e.g., MHC class I) sensed by the adaptive and innate immune systems, and through triggering epithelial-to-mesenchymal transition in tumor cells that are more metastatic and have tumor stem cell-like properties.

2.3.1 Immune System as a Driver of Melanoma Initiation

It has long been known that chronic inflammation through innate immune system mediators regulating wound healing can drive cancer development and initial progression. Cytokines such as TNF- α , IL-1 α , IL-1 β , and IL-17 and its associated cytokine IL-23, as well as IFN- γ , are key players regulating both CD8⁺ CTL and CD4⁺ T-helper responses that can drive tumor initiation [45–48]. These cytokines (TNF- α , IFN- γ , IL-1 α / β) are not only products of adaptive immune responses but

can also be produced by innate immune system cells such as macrophages, granulocytes, and NK cells homing to damaged or stressed tissues [45].

Building on our theme of cancer as a “wound that never heals,” growing data in melanoma indicates that UV radiation-damaged melanocytes and keratinocytes in the skin can trigger a stress response that activates an innate inflammatory response through the influx of innate immune cells but at the same time triggers the release of mediators, such as IL-10, that inhibit DC activation and initiation of an adaptive immune response [49]. This may also be a protective mechanism to prevent immunopathology at the stressed site, but, in terms of cancer initiation, this protective pathway “does not know” that a cancerous situation is causing the initial stress. For example, in a recent study by Zaidi et al., IFN- γ was found to be a key mediator in cutaneous melanoma initiation after UVB irradiation in a new hepatocyte growth factor/scatter factor transgenic mouse model [50]. UVB, but not UVA irradiation of neonatal pups, led to melanocytic tumors reminiscent of human melanoma that was prevented by blocking IFN- γ , but not type I IFN [50]. The source of IFN- γ facilitating tumor formation was found to be from activated CCR2 chemokine receptor-positive macrophages infiltrating into the damaged skin area via localized production of CCR2 ligands [50]. IFN- γ -positive macrophages were also found in >70 % of human melanoma examined [50]. IFN- γ and IL-12 as innate immune system drivers can also lead to the activation of other “bystander” macrophages and myeloid cells to express inducible nitric oxide synthase (iNOS) releasing nitric oxide (NO) that together with reactive oxygen species (ROS) can cause a highly reactive compound, peroxynitrite, that causes DNA damage and protein tyrosine nitration [51–55]. This together with other mutations in melanocytes induced by UVB-induced DNA damage can set off a chain reaction of events that leads to more genetic damage, more inflammation, and then cancer formation. Activated macrophages also produce IL-6, IL-1, and TNF- α , which activates NF- κ B in transformed cells protecting cells from apoptosis. In fact, activated macrophages (that also increase with aging in humans) are drivers of many types of cancer initiation not just melanoma; underscoring how chronic inflammation during aging (also called “inflammaging”) is a major driver of cancer [56–58]. For further information and concepts of the role of inflammation and cancer, an excellent review on the role of inflammation as a driver of cancer initiation has recently been published by Trinchieri and colleagues [45, 59].

2.3.2 *Cancer Immunosurveillance*

The notion that the immune system can detect transformed cells in early subclinical cancerous lesions has been suggested for over 100 years and was first formulated into a working hypothesis by Macfarlane Burnet in the 1950s as the “cancer immunosurveillance hypothesis” [60]. Cellular transformation induces cell stress and it was postulated that ligands appear on the surface of transformed cells that are recognized by innate immune cells such as NK cells and macrophages for removal of these cells by cytotoxic killing and phagocytosis. With the discovery of adaptive

immunity and the model of clonal selection in the 1950s, Burnet proposed that antigen-specific T cells recognize mutated antigens or altered self-antigens in these early transformed cells and killed these cells off in most cases before tumor arises and that only under conditions of immunosuppression or compromised antigen-specific lymphocyte function would cancer arise [60, 61]. Thus, the notion of the cancer mutanome (of huge interest currently in tumor immunology) is not new, but back then the molecular tools, including knowledge of the TCR and MHC restriction, was not yet discovered. It was during this time in the 1960s that Jacques Miller (also at the Walter and Eliza Hall Institute with Burnet) discovered that T lymphocytes differentiated in the thymus gland and that athymic animals or early thymus removal obliterated T-cell development [62]. The cancer immunosurveillance hypothesis was debated and a number of indirect sources of evidence were used to support it, such as the increased incidence of cancer with aging due to the successive loss of immunocompetence, the development of cancer under other immunocompromised situations such as after organ transplantation and the development of secondary cancers in individuals treated with high-dose chemotherapy for a previous cancer, and autopsy data that found that many women and men have latent, preinvasive ductal breast carcinomas and in situ prostate carcinomas that seemed to have remained dormant. Fast forwarding to the 1970s, a problem emerged with the introduction of athymic nude mice that were thought to completely lack any T cells (the first immunodeficient mouse model available as a result of a random mutation during inbreeding) that set the field back unfortunately for more than a decade due to unsubstantiated dogma that arises in the scientific community. Here, in a number of careful longitudinal studies in nude mouse strains, Stutman and colleagues found that the incidence or rates of spontaneous cancers, including leukemias, lymphomas, and virally induced sarcomas and sarcomas induced by methylcholanthrene (MCA), were not increased relative to non-immunocompromised mice, including those syngeneic with nude mouse strains [63–65]. This seemed to cast the death knoll for the immune system as being a sentinel against cancer until further research on nude mouse strains in the 1990s after the discovery of NK cells (MHC nonrestricted effector cells) found that NK cells exist in relatively normal numbers and activity in nude mice [66–68]. Moreover, a considerable amount of extrathymic T-cell differentiation (e.g., gut-associated lymphoid system) was found to occur in aged nude mice indicating that the nude mouse was a bad model to address the cancer immunosurveillance hypothesis [66–68]. Actually, this was already hinted at by a paper by Stutman himself in 1975 showing delayed tumor appearance in older nude mice in comparison with younger nude mice after murine sarcoma virus infection that was mediated by transfer of splenocytes from the older nude mice into the younger ones [64].

The development of gene knockout mouse strains in the 1990s allowed researchers to determine whether specific cell types and cytokines play a role in the antitumor immune response and whether immunosurveillance of early preneoplastic and neoplastic lesions occurs. In addition, monoclonal antibodies specific for cytokines and cell surface proteins became available that could either block the effects of specific cytokines or deplete specific lymphocyte subsets *in vivo* through

complement-mediated or antibody-dependent cellular cytotoxicity (ADCC)-mediated pathways. Using these approaches, the group of Rob Schreiber began to elegantly resurrect Burnet's original idea using a spontaneous sarcoma tumor model arising in mice after carcinogen treatment with the carcinogen MCA [69, 70]. Using this model they showed an increased MCA sarcoma incidence in mice lacking type II IFN (IFN- γ) as well as STAT1 the key transcriptional factor activated downstream of IFN- γ and type I IFN signaling. Similar results were found by injecting blocking IFN- γ antibodies. Later, a more immunodeficient mouse model lacking the recombina-activating gene (RAG) preventing all T- and B-lymphocyte development was also found to have increased incidence of both MCA-induced sarcoma and another spontaneous tumors. Similar results were obtained with mice lacking NK T cells [71–75].

Another important concept emanating from these studies was “immunoediting”: the notion that the adaptive immune response can control tumor growth, especially early on, but also selects for tumor cell variants in the primary tumor that are resistant to the T-cell response as a result of antigen loss or other immunosuppressive mechanisms. Thus, the immune system “immunoedits” or “immunoselects” resistant tumor variants that can grow faster and evade further adaptive T-cell responses [71–74]. Work showing that tumors developing after MCA treatment in RAG knockout mice grow slower and many times can even be rejected when transplanted into wild-type syngeneic mice while those originating in wild-type mice grew faster when transplanted into other naive wild-type mice and were almost never rejected elegantly gave proof to this concept [71–74]. Since then, many groups are now studying the molecular mechanisms of this immunoediting process. As discussed below, this concept is also emerging to be critical at all stages of cancer where the immune system is constantly engaging tumors throughout the body and placing constant “immune pressure” on tumors leading to selective survival of resistant tumor stem-like cells either as a result of acquired or in intrinsic mechanisms.

2.3.3 Immunoselection of Resistant Tumor Subtypes by the Innate and Adaptive Immune Response

A number of studies in animal tumor models of melanoma using the B16 tumor cell line as well as spontaneous animal tumor models employing the *BRAF*^{V600E} oncogene together with PTEN knockdown specifically in melanocytes [using a tyrosinase or dopachrome tautomerase (DCT) promoters] have shown that the adaptive immune response leads to tumor antigen escape variants and preferential survival melanoma tumor cells with cancer stem cell properties. For example, in a recent study using the B16 model, adoptive cell therapy with melanoma-specific T cells was found to elicit a down-modulation of melanoma tumor antigen expression (MART-1, tyrosinase, and gp100) mediated through a mechanism involving tumor-localized TNF- α [76]. This led to renewed tumor growth. However, the loss of melanoma tumor antigen expression was transient and not permanent such that cessation

of the immune response or neutralization of TNF- α resulted in tumor antigen re-expression [76]. Thus, proinflammatory cytokines like TNF- α released during an antitumor adaptive immune response can directly down-modulate antigen expression. The mechanism behind the transient nature of this process will need to be worked out but may be associated with an inflammation-induced dedifferentiation phenomenon or the selective survival of tumor cells with stem-like properties that lack tumor antigen expression.

This latter scenario is now gaining traction in the field in recent studies using an anti-melanoma vaccine model in which an incomplete antitumor immune response led to the preferential survival of melanoma stem-like cells that have lost antigen expression as well as other cell adhesion molecules (e.g., E-cadherin and claudin family tight junction protein) in a process that is beginning to be called “pseudo-epithelial-mesenchymal transition” (pseudo-EMT) after the well-documented EMT events that occur in epithelial malignancies [77–80]. These concepts have been elegantly studied by Vile and colleagues who used a B16 melanoma cDNA library-based vaccine approach using the vesicular stomatitis virus (VSV) vector [81]. When this melanoma VSV-cDNA vaccine was given in a suboptimal way so that an incomplete tumor eradication is ensued by limiting the number of booster inoculations of the vaccine, established tumors shrank, but then regrew after a while. Analysis of these early regrowing tumors revealed that they were composed of these pseudo-EMT melanoma cells that could be cultured as nonadherent, suspended cells in much the same way as tumor stem cells from epithelial cancers like breast, lung, and prostate cancer. These stem-like melanoma cells grew as nonadherent spheres (so-called melanospheres) and were tumorigenic when transplanted into naive mice [81]. However, as found in the study mentioned above, the phenotype of these stem-like cells were not stable; after 1–2 weeks in culture, they became adherent again and could be treated again with a new VSV-cDNA vaccine [81]. This underscores an interesting concept that the immune system may place a transient, but not permanent, selective pressure on melanoma tumors inducing phenotypic alteration that evades the immune response, but not a permanent genetic change. This is good news for immunotherapy, but also suggests that we need to be highly cautious. Immunotherapies, such as vaccines and adoptive cell therapy that do not induce a strong enough response eradicating the tumor completely or invoking an irreversible dormant state, may actually select for this pseudo-EMT phenomenon in melanoma manifested by the survival of melanoma stem-like cells that can migrate to other areas of the body or eventually regrow at the original tumor sites. These stem-like cells have also been found to be slowly dividing (so-called slow cycling cells exhibiting high levels of mitochondrial respiration and are resistant to chemotherapy or targeted drugs (e.g., MAPK pathway inhibitors) aimed at inhibiting rapidly cycling tumor cells [82].

The exact molecular mechanisms of this induction of this resistant transient melanoma stem-like cells and what cytokines or other signaling pathways are involved will need to be investigated. Nevertheless, it emphasizes the dynamic nature of the immune system-tumor interaction and how the immune system, via proinflammatory signaling pathways, can lead to an “adaptive resistance” of the tumor against further immune attack. Recently, this has been elegantly demonstrated by the role

of adaptive immune cytokines, such as IFN- γ , at the tumor site inducing the expression of immunosuppressive molecules by tumor cells (e.g., PD-1 ligand) that then feedback inhibits the local effector T-cell response [20].

2.4 Evolution at Play: Counterbalancing Immune Regulatory Factors in Melanoma Through “Adaptive Resistance”

As part of the different strategies to escape the immune system, the melanoma tumor microenvironment has evolved to suppress its action both locally and systemically (see Fig. 2.3 for a summary of the major mechanism discussed in this section). This includes utilizing suppressive cells within the immune system as well as directly through the tumor interaction with local T cells. Suppressive immune cells that are found within and around the tumor include MDSCs, T-regulatory cells

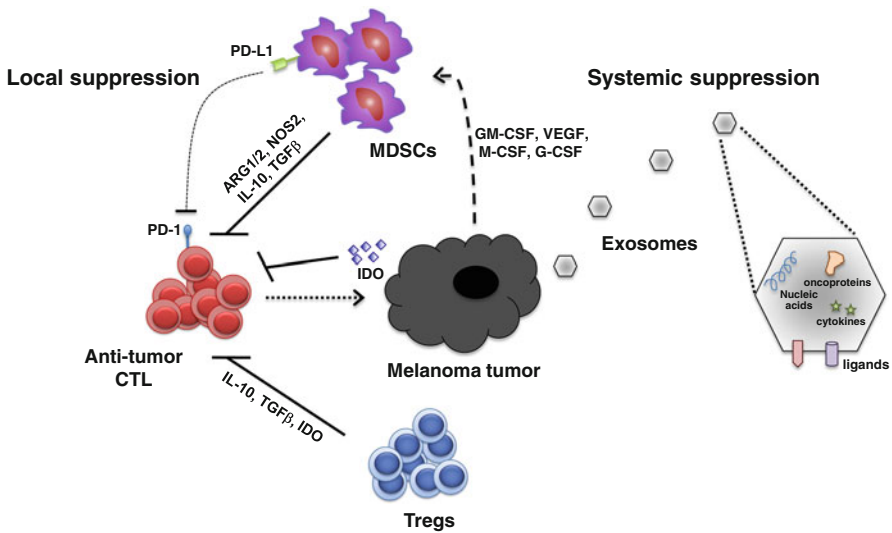


Fig. 2.3 Mechanisms of tumor-induced immunosuppression and adaptive resistance. Suppression exerted on the immune system by the tumor can be both local and systemic in nature. Locally, tumors can directly suppress invading antitumor CTL through the production of factors such as IDO (indoleamine 2,3-dioxygenase) or indirectly via the recruitment of suppressive immune cells such as Tregs and MDSCs. These cells, in turn, can mediate suppression through the production of cytokines such as IL-10 and TGF- β as well as through the production of arginase 1/2 (ARG1/2) and nitric oxide synthase 2 (NOS2). Direct suppression can also be mediated by the expression of inhibitory ligands such as programmed death ligand 1 (PD-L1) whose receptor, programmed death 1 (PD-1), is expressed on activated T cells. Tumors can induce systemic suppression via the production of exosomes expressing inhibitory ligands and containing suppressive cytokines, oncoproteins, and nucleic acids. Exosomes are able to help generate a “tumor-friendly” environment allowing for metastasis and promoting tumor growth

(Tregs), and type II CD4⁺ T-helper cells (Th2 cells). Some major mechanisms of T-cell suppression by MDSCs involve their production of arginases 1 and 2 (ARG1 and ARG2) and nitric oxide synthase 2 (NOS2) as well as nitric oxide (NO) and ROS and peroxynitrite. Both ARG1 and ARG2 as well as NOS2 synthesize L-arginine and block translation of a component of the TCR-signaling complex (the CD3 ζ chain), inhibiting T-cell proliferation and inducing T-cell apoptosis [83, 84]. Peroxynitrite is the product of nitric oxide and superoxide anion. Increased levels of peroxynitrite have been correlated with a bad prognosis in multiple types of solid tumors including melanoma [55]. Peroxynitrate production by MDSCs within the immediate environment of the T cells has been shown to induce nitration of the TCR and CD8 molecules, thereby affecting their specific peptide-binding ability, resulting in the T-cell failure to respond to antigenic stimulation [85]. MDSCs can also suppress activated CTL, macrophages, and NK cells within the tumor microenvironment through secretion of suppressive cytokines such as IL-10 and the expression of membrane-bound TGF- β [86, 87]. Immature myeloid cells (IMCs) undergo differentiation into MDSCs in response to cytokines and other host factors that are released during cancer and some infections. MDSCs are induced by the tumor microenvironment due to the inflammatory nature of the tumor site. Tumor cells have been shown to produce the cytokines GM-CSF, G-CSF, M-CSF, and VEGF which all favor MDSC development [88]. In addition, stromal cells, activated T cells, and macrophages within and around the tumor produce other cytokines such as IL-1 β , IL-4, IL-13, IL-6, IL-10, IFN- γ , and PGE that all aid in the generation of MDSCs from normal circulating IMCs [88].

As mentioned above, Tregs can be found within the melanoma tumor microenvironment and are a major factor mediating immunosuppression. Tumor production of the chemokines CCL2 and CCL22 has been shown to recruit Tregs to the tumor site, driven by mediated expression of the chemokine receptor CCR4 by the Tregs [89, 90]. At the tumor site, Tregs are able to suppress activation and proliferation of T cells indirectly through their secretion of IL-10 and TGF- β . Some studies have shown that Tregs are able to mediate direct suppression of activated T cells via cell-to-cell contact. Recently, the Whiteside group demonstrated that Tregs are in fact able to kill CD8⁺ T cells through Fas-mediated apoptosis in cancer patients [91]. The different subsets of Tregs within the tumor microenvironment may also be important to different degrees. For example, it has been demonstrated in melanoma that expression of ICOS marks a subset of highly activated Tregs found at the tumor site but not in the blood that are able to mediate stronger suppression than their ICOS^{lo} counterparts [92]. Tregs are also able to inhibit activated effector cells through their generation of adenosine via CD39 and CD73. CD39 converts adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine monophosphate (AMP). CD73, in turn, degrades AMP into adenosine. ATP is released by damaged or dying cells and has a proinflammatory effect on the innate immune system [93]. Conversely, the presence of adenosine induces the expression of adenosine receptors on local cells including activated effector T cells and induces energy [94].

Not only does the tumor induce inactivation of antitumor CTLs by the body's own immune system, the tumor cell itself utilizes inhibitory molecules expressed by the CTL to induce further suppression/inactivation. Two major pathways that have been extensively studied are the CTLA-4/CD80/CD86 and the PD-1/PD-L1/PD-L2 inhibitory pathways. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is upregulated on T cells following activation and is also expressed on Tregs. CTLA-4 provides a natural break for immune activation as it displays a high affinity for the costimulatory ligands CD80 and CD86 (expressed on APCs) effectively blocking the ability of the T cells to receive stimulation through CD28. In fact, a recent study has shown that CTLA-4 actively pulls CD86 from the surface of an APC and internalizes it, removing the ability of the APC to costimulate reactive T cells [95]. However, the expression of CD80 or CD86 on the tumor cells has not been shown to be induced in response to the presence of T cells in or around the tumor.

Conversely, another inhibitory molecule that is upregulated on T cells following activation, programmed death receptor 1 (PD-1), is found to be highly expressed in T cells within melanoma tumors. PD-1 has two known ligands, PD-L1 and PD-L2. Expression of PD-L1 is induced on the tumor cells due to the presence of the inflammatory cytokine, interferon-gamma (IFN- γ). Ligation of PD-1 by PD-L1 or PD-L2 results in a strong brake on the T cell in terms of cytokine secretion, proliferation, and killing potential. Recently, it was found that expression of PD-L1 by the tumor occurs after tumor invasion by T cells in direct response to IFN- γ production by T cells [20]. Thus, the tumor is able to respond to the presence of activated T cells producing inflammatory cytokines and mediate their suppression. Other immune modulatory molecules are expressed by the melanoma tumor and tumor-associated antigen-presenting cells including herpes virus entry mediator (HVEM). HVEM interaction with B and T-lymphocyte attenuator (BTLA) on T cells results in suppression of cytokine production and proliferation. Melanoma cell expression of HVEM has been shown to mediate direct suppression of activated CD8⁺ T cells [96]. While HVEM is not expressed on normal melanocytes, it remains unclear what factor induces its expression by melanoma tumor cells.

Another major mechanism of tumor-driven immunosuppression is the secretion of indoleamine 2,3-dioxygenase (IDO) by the tumor cells. IDO is an enzyme that catalyzes tryptophan degradation. T cells undergo cell cycle arrest in the absence of tryptophan. This mechanism for local immunosuppression was first described in mouse models and human xenograft models. This study showed that most human tumors express IDO and that T cells fail to proliferate and reject such tumors in mouse [97]. More recent work has shown that the presence of IDO in the tumor is a direct result of IFN- γ production by CD8⁺ T cells [20]. Furthermore, the presence of IDO in melanoma lymph node metastases has been shown to correlate with a high level of Treg infiltration and decreased survival [98]. Thus the tumor is responding directly to the attack by the immune system by producing factors and expressing ligands that in turn aid in immunosuppression.

Melanoma tumors are also able to suppress the immune system systemically through the release of exosomes carrying proteins, miRNAs, and mRNA that circulate through the blood [99–101]. Exosomes are small vesicles naturally released by

the tumor. Initially this was considered a random process but recent data has suggested that, instead, this provides a way for the tumor cells to communicate with each other as well as educate niches to be permissive to the development of metastases [102, 103]. In fact, exosomes have been shown to carry oncoproteins such as the MET protein, which in turn induces secretion of VEGF and prepares the environment for metastatic development [103]. Exosomes are able to influence a permissive immune niche for tumor growth through a skewing of the development of myeloid cells toward the MDSC state through delivery of cytokines and other key factors involved in their development to distant hematopoietic sites in the body [104].

Although the tumor microenvironment has evolved to aid its own growth and development through taking advantage of features of the immune system, many emerging therapies are counterbalancing the immune response against the tumor. Adoptive T-cell therapy, blockade of inhibitory signaling pathways through the use of blocking antibodies, and the addition of cytokines to aid the growth and survival of tumor reactive T cells will all be discussed in a later section.

2.4.1 Immunotherapy: Enhancing De Novo Antitumor Immunity

Until recently, treatment options for solid tumors such as melanoma were surgery, chemotherapy, and radiotherapy. Although these treatments can be very effective, some patients affected by aggressive cancers such as metastatic stage IV melanoma often have to face the failure of treatment. In the past three decades, a new type of treatment using biological resources has come to the forefront of cancer therapy, immunotherapy. Immunotherapy capitalizes on the use of different components of the immune system such as antibodies and immune cells to eradicate the tumor cells. This concept is based around the fact that the tumor expresses TAA, as previously discussed, and so it is seen by the immune system as a foreign object that needs to be eliminated. Immunotherapy is also about providing tools to activate the immune cells and/or protecting them against suppression generated by the tumor and/or its environment. Immunotherapy of cancer can be divided into four categories: cytokines, vaccines, antibodies, and cell based. This section will generally address these different categories specifically regarding melanoma treatment.

Melanoma has been considered the pioneer cancer regarding development of immunotherapy due to the richness of TAA-specific CD8⁺ T cells found in melanoma patients' tumors and blood [105, 106]. One of the first types of immunotherapy to be applied was the use of cytokines as sole agents. The immune response is a complex sequence of events that requires communication between the immune cells and the surrounding environment. Cytokines are small proteins that play an important mediator role in the communication network. There are over 200 known cytokines involved, both locally and systemically, in different spheres of the immune response. The specificity of the cytokine response is ensured by the expres-

sion of the corresponding receptor(s) on the surface of the target cell. At the site of secretion, the gradient created and the short half-life of cytokines also dictates the response. Capitalizing on the importance of cytokines in the mediation of the immune response, IL-2 was the first to be used as a systemic treatment in melanoma. An antitumor response was observed in these patients but only 3–5 % achieved a complete and durable response [107, 108]. Aside from activation of antitumor T cells, administration of systemic IL-2 can also lead to the activation of Tregs, which express a receptor that is highly specific for that cytokine [109]. Activation of these Tregs can block the antitumor response, diminishing the effect of the therapy. Another side effect of IL-2 therapy that is encountered, especially when using high doses, is the vascular leak syndrome that occurs with a massive release of cytokines such as IL-1, IFN- γ , and tumor necrosis factor alpha (TNF- α). These cytokines interfere with vascular permeability, which can result in serious complications for the patient such as pulmonary edema [110]. Renal, gastric, and neurologic toxicities have also been reported in patients treated with high doses of IL-2 [111]. In an effort to avoid such side effects, different groups have been working toward developing synthetic or chimeric versions of this cytokine, which represents a promising avenue for immunotherapy, especially if used in combination with the other types of immunotherapy subsequently described here [112–115]. Aside from IL-2, which has so far been the most popular cytokine to be used as a front line treatment, other cytokines such as IFN- α , IL-12, IFN- γ , TNF- α , GM-CSF, IL-15, and IL-21 have either been tested or are currently being used in ongoing clinical trials [116, 117] and www.clinicaltrials.gov. These cytokines have been used either in a recombinant form, as a fusion protein, or in a gene vector.

Another type of immunotherapy that has been widely tested in metastatic stage IV melanoma is the peptide vaccine. Capitalizing on the innate presence of T cells with directed specificity against defined MHC class 1 peptides derived from TAA such as MART-1, tyrosinase, and gp100, clinical trials have been carried out with vaccines containing these peptides with the addition of Montanide ISA (*incomplete Freund's adjuvant*) and cytokines such as IL-2 to boost the patient's endogenous antitumor response. Aside from reports of higher levels of antigen-specific T cells in the blood post-vaccination, no significant clinical response was obtained. However, one study using a gp100 peptide reported a higher clinical response when compared to IL-2 alone and brought hope for this cost-effective strategy [118]. However, questions have been raised regarding the use of other types of peptide such as those generated from mutations and identified by exome sequencing of the tumor. It is believed that these peptide could be able to raise a more potent and durable endogenous antitumor response [38, 119]. In addition, questions have also been raised around the use of Montanide as an adjuvant as it may not be optimal to generate potent immune responses at the tumor site [120]. Research efforts are now being pushed toward the development of new strategies to enhance immunogenicity of peptides with the use of new adjuvants such as crystals or viral plant particles. APC subsets, such as DC, have also been used in combination with peptides or the whole protein to help boost the endogenous antitumor response by delivering the antigen to the tumor site and providing additional costimulation to T cells. Our

group is currently conducting a clinical trial combining MART-1 peptide-loaded DC, high-dose IL-2, and adoptive transfer of TIL which will be discussed later in this section. Other trials using peptide-pulsed DC or engineered DC-expressing TAA are also currently ongoing (www.clinicaltrials.gov). Although promising, there is still much debate about the optimal type of DC to be used in this vaccine setting [121].

Tumor cells have also been used in vaccination. Whole cell lysates, generated by multiple rounds of freeze/thaw to generate tumor necrosis or irradiation to induce apoptosis, have been given as a sole agent or combined with the administration of preloaded or fused DC (www.clinicaltrials.gov). Although this approach provides access to a myriad of different known and unknown TAAs to be included in the vaccine, no major benefit was reported for melanoma. Interestingly, other cancer cell components, such as exosomes [122–124], are also being explored for use in a vaccine. Melanoma tumor cells have the capacity to secrete microvesicles called exosomes, which may contain TAA such as gp100, MART-1, and tyrosinase [125]. These exosomes can be used as components of an antitumor vaccine to boost the immune response. However, one must be careful with the use of naturally derived exosomes for it has been shown that these microvesicles can contain immunosuppressive factors and even receptors, which could suppress the immune response [122–124, 126]. They can also contain oncoproteins such as MET which is an oncogenic tyrosine kinase receptor, which can favor metastasis and induce vascular leakage [102, 103].

As described in previous sections, the tumor microenvironment takes advantage of the presence of inhibitory molecules on both immune and cancer cells to suppress the antitumor immune response. Conversely, this also provides a target to block these immune checkpoints. Current methods use antibodies directed against inhibitory molecules found on the invading T cells such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death receptor 1 (PD-1). Another method is designed to block the inhibitory ligands found on the tumor cells and the MDSCs within the tumor microenvironment such as programmed death ligand 1 (PD-L1). Anti-CTLA-4 (drug name ipilimumab, marketed by Bristol-Myers Squibb as Yervoy) was the first checkpoint inhibitor to gain FDA approval for the treatment of melanoma. In a recent melanoma phase III clinical trial, patients who received anti-CTLA-4 showed an improved overall survival of 10 months had an overall response rate of 10.9 with 60 % maintaining an objective response rate for 2 years post treatment [127]. While anti-CTLA-4 has been proven effective in some patients, the side effects can be severe. The use of this antibody causes the breakdown of immune tolerance to normal body tissues resulting in an increased risk of autoimmunity as well as gastrointestinal toxicities, hepatitis, and dermatitis.

Antibodies directed against PD-1 (nivolumab and MK3475) have also shown considerable efficacy in clinical trials in melanoma as well as in other types of cancer. The Bristol-Myers Squibb anti-PD-1 antibody (nivolumab), which blocks the binding of PD-1 to both PD-L1 and PD-L2, showed significant effectiveness in late-stage melanoma with a 31 % response rate and median response duration of 24 months [128]. Blockade of PD-1, using another antibody generated at Merck

(lambrolizumab), resulted in a 38 % overall response rate with 81 % durable response at 11 months after initiation of treatment of stage III/IV melanoma [129]. Interestingly, the adverse side effects of anti-PD-1 therapies are not as severe as those using anti-CTLA-4. Although the risk of autoimmunity is still present in anti-PD-1 therapy, most patients experienced rash, diarrhea, and fatigue.

The use of antibodies directed against PD-L1 has been less utilized in the treatment of melanoma than the anti-PD-1 therapy but still shows therapeutic efficacy. Cellular inhibition through PD-1 can be due to its binding either PD-L1 or PD-L2. Thus blocking PD-1 in fact blocks both these pathways, while PD-L1 blockade only blocks the one. However, 2 independent phase I/II dose escalation trials have demonstrated that treatment of melanoma with anti-PD-L1 results in 17 % (BMS-936559) and 38 % (MPDL3280A) response rate [130]. Such positive results have increased the interest in the use of antibodies as therapeutic agents in melanoma.

Alternatively, antibodies directed against important T-cell costimulatory molecules such as OX40 and 4-1BB are regaining ground in the treatment of melanoma. In this case, however, the antibodies are agonistic in nature and so induce a positive feedback signal to the T cells. The combination of anti-OX40 and IL-2 has been shown to be effective in mouse models [131]. The combination of anti-OX40 and anti-CTLA-4 therapy in metastatic melanoma is also being examined in a current phase I/II clinical trial. In addition, different strategies targeting 4-1BB are emerging to be potentially effective.

While the methods described above are able to induce tumor regression in some patients, currently some of the most effective treatments for late-stage melanoma are cell based and referred to as adoptive cell therapy (ACT). This involves the isolation of TAA-specific T cells from the patients' blood or tumor. As a number of melanoma TAA are characterized (e.g., MART-1, gp100), these cells can be isolated from the blood and expanded *in vitro* in an antigen-specific manner and then infused back into the patient. This method has been tested using CD8⁺ T-cell clones (homogenous population) directed against MART-1 and gp100 MHC class I peptides. One study from 2002 reported tumor regression in 2 out of the 10 patients infused with the TAA-specific clones [132]. Subsequent work revealed that persistence of the transferred cells and gain of a memory phenotype after infusion were important to achieve clinical response [133]. The use of selected CD4⁺ T-cell clones directed against the NY-ESO1 antigen in melanoma has also been tested but with little reported success [134]. In addition, the identification of other epitopes to generate CD4⁺ T-cell clones is challenging and so does not play in favor of the use of that subset in ACT [135].

Expanding TAA-specific T cells of known reactivity is limited by the number of known epitopes and only provides for reactivity against those epitopes for a defined MCH-class I type, the most popular being HLA-A0201. Another cell-based approach is based on the concept that the T cells found at the tumor site will be enriched for reactivity against multiple known and unknown TAAs. This involves the surgical resection of a tumor and expansion of the tumor-infiltrating lymphocytes (TILs) followed by infusion into the patient. Adoptive transfer of TIL for the treatment of metastatic melanoma was first performed at the National Cancer

Institute [136]. In this study, TIL ACT was followed by IL-2 therapy to aid T cell growth *in vivo*. By 1994, TIL ACT in metastatic melanoma had a response rate of 34 % [107]. In the years that followed, it was found that non-myeloablative chemotherapy prior to TIL ACT improved the response rate to around 50 % at three independent institutions [137–139]. Lymphodepletion prior to TIL transfer aids in the removal of suppressive immune cells such as Tregs and provides access to endogenous cell survival cytokines such as IL-7 and IL-15. Interestingly, while the presence of a high percentage of CD8⁺ T cells in the infusion product was found to significantly correlate with clinical response [138, 139], infusion of CD8⁺ T cells alone did not improve clinical efficacy above the transfer of bulk TIL [140]. However, the use of total body irradiation prior to TIL infusion was successful in increasing the response rate up to 72 % [137].

Given the success of TIL in the treatment of melanoma, some researchers have attempted to manipulate the TIL to enhance their antitumor functions and persistence after treatment through the secretion of cytokines (e.g., IL-12) [141]. Current clinical trials at MD Anderson are exploring whether inducing expression of a chemokine receptor (CXCR2) might aid in the migration of TIL to the tumor site or make the TIL unable to be suppressed by secreted factors (DN TGF- β R).

TCR gene therapy is another way to induce an antitumor response that has been tested using PBMCs virally manipulated to express a foreign TCR to induce recognition of known TAAs such as MART-1 [142]. In early clinical trials, only two patients showed regression of tumor metastases and had persistence of the infused, genetically engineered T cells at 1 year after infusion. The possibility of engineered TCR signaling not being powerful enough to generate an antitumor response and persistence was suggested and so a new TCR was generated against MART-1. This new TCR demonstrated a higher affinity for the MART-1 peptide and was used to treat an additional 20 patients followed by IL-2 with prior lymphodepletion. While a 30 % objective clinical response was observed, some patients experienced a strong autoimmune response such as hearing loss due to the destruction of normal melanocytes in the ear as well as in the skin and eye [143]. A recent study using anti-MAGE-A3-engineered T cells showed clinical effectiveness but also generated severe neurologic toxicities resulting in the death of two patients [35]. Overall, these studies highlight the powerful responses generated by the use of the engineered TCRs showing a strong affinity for a specific peptide and are making the field aware of the importance of selecting the right TAA to target to avoid dramatic off-target consequences.

Chimeric antigen receptors (CARs) represent another mechanism by which PBMCs and potentially TIL can be manipulated to express selected anti-TAAs. CAR T cells are generated to express a monoclonal antibody as a receptor to recognize a given TAA. This allows the T cell to have reactivity against tumor antigens that does not require processing and presentation of the antigen, hence no MHC restrictions. CARs have been very popular in treatment of some hematologic cancers with the last couple of years demonstrating very promising results [144–146]. CAR-engineered T cells are a powerful tool by which it is possible to generate a strong immune response; however, studies in solid tumors other than melanoma using an anti-erbb2 CAR have also shown that there is great risk associated with

such a high potent response [147]. Again, precaution has to be taken in the selection of the target and its expression in normal cells. Mechanisms for quick destruction of the CAR-expressing cells are also being developed to eradicate the cells if needed. Still, clinical trials using anti-VEGFR-engineered CAR T cells are currently recruiting patients with stage IV melanoma (www.clinicaltrial.com). Additionally, based on preclinical data showing anti-melanoma tumor responses and increased survival in mouse xenograft models, other clinical trials are recruiting patients for treatment using anti-GD2 and anti-GD3 CAR T cells in solid cancer [148, 149].

The cellular-based immunotherapies are a great example of how the combination of treatments can be used to obtain clinical success. Transfer of TAA-specific cells combined with cytokines and sometimes booster vaccines has been achieving better results in clinical trials than cell product transfer alone. Antibodies like anti-CTLA-4 and anti-PD-1 are now being added to the treatments to improve therapy. As single-therapy agents, the new compounds do not always show the expected results; however, their use is setting the stage for the combination of multiple targeted agents as well as a combination of cell-based and antibody-based immunotherapy. Many questions remain regarding when and which immunotherapy should be applied for the treatment of melanoma. The use of biomarkers to aid in this decision-making process is attractive but remains unclear. For example, in an anti-PD-1 study, the presence of PD-L1 in the tumor emerged as a biomarker for response to therapy [128]. However, this has not yet been confirmed in other studies. Another example is the presence of TIL in the tumor as a biomarker for ACT. If it was possible to know prior to resection if the tumor is infiltrated with lymphocytes and their functional quality, we could know whether TIL ACT is appropriate and if not which other treatment to use [150]. One thing is clear; immunoselection is one of the worst enemies of immunotherapy. This is why it is vital to target multiple tumor antigens and combine cell-based and antibody-based therapy to maximize antitumor responses and persistence of TAA-specific cells.

2.5 Biomarkers of Active and Suppressive Immune Regulatory Mechanisms in Melanoma Patients: Is the Presence of Immunosuppressive Factors in the Tumor Microenvironment Bad or Good?

More and more evidences emerged and showed that the classification of cancer related to estimating the clinical outcome should base on the tumor microenvironment, considering the effects of the host immune response. The immunological status of the microenvironment among different immunotherapies could explain the difference between response and resistance of melanoma patients or predict patient prognosis. Currently, there are several approaches to study or measure the role of the immune system in melanoma. As shown in the model in Fig. 2.4, key biomarkers can be measured in an interaction between tumor- and host-derived factors regulating the degree of T cell infiltration into tumors and the phenotype and antitumor

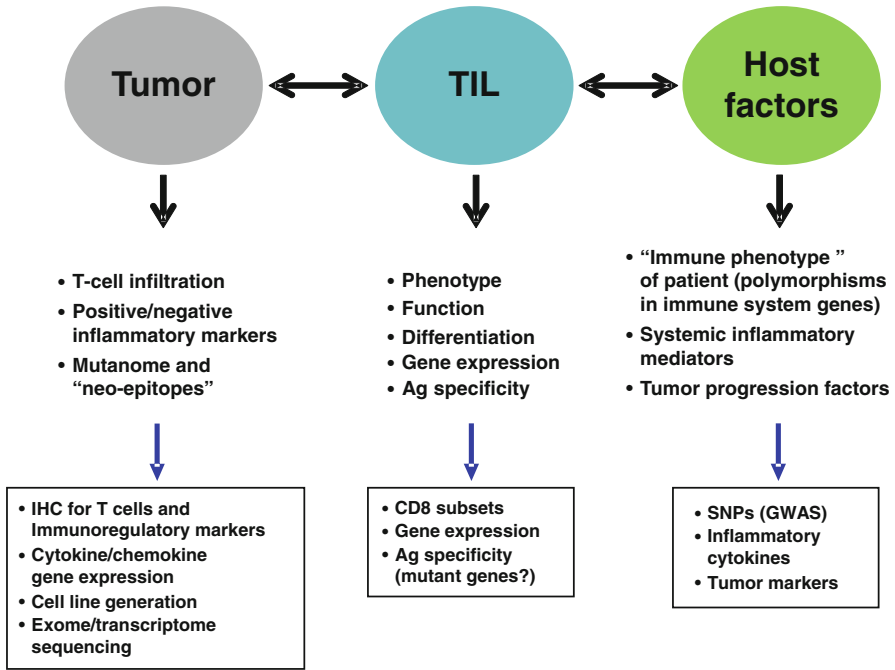


Fig. 2.4 Systems biology approach to biomarker discovery for melanoma immunotherapy. Biomarkers can be used to measure the interaction between tumor- and host-derived factors and the regulation of T-cell infiltration into tumors and the phenotype and antitumor activity of the TIL. This approach may allow physicians to delineate patients who may respond to immunotherapy prior to therapy

activity of these TILs. It can delineate patients who are responding to immunotherapy as well as predicting who may respond before therapy.

Gene expression profiling using Nanostring nCounter™ analysis system could accurately quantify the RNA levels from fresh-frozen and FFPE samples in small amounts of total RNA [151]. This new technology could identify different gene panels, such as immunology panel, inflammatory panel, and cancer-related gene panel, as potential immunotherapy targets for melanoma [152] or as biomarkers to predict melanoma patient prognosis, and therefore identify the patients who would benefit from immunotherapy. Messina et al. found that 12-chemokine (CCL18, CCL19, CCL2, CCL21, CCL3, CCL4, CCL5, CCL8, CXCL10, CXCL11, CXCL13, and CXCL9) gene expression signature (GES) score was associated with better overall survival in metastatic melanoma [30]. Same results were shown by Tanese et al. when they compared the gene expression profile between iNOS-positive and iNOS-negative tumor samples and found CXCL10 expression was upregulated in iNOS-negative groups and had the most favorable prognosis [153], while inducible nitric oxide synthase (iNOS) and nitrotyrosine (NT) expression in patients with stage III melanoma strongly correlated with poor survival [55]. Although *BRAF* V600E, *KIT*, and *NRAS* mutations are the most important drivers of melanoma

development and could predict the poor outcome of melanoma patients [154], epigenetic analysis found the DNA methylation is associated with melanoma progression [155, 156].

Patients with a high ratio of T-cell infiltration have clinical responses to melanoma vaccines and anti-CTLA-4 monoclonal antibody (ipilimumab) therapies [157, 158]. Steven A. Rosenberg's group also found an overall increase of total number of CD8⁺ T-cell infiltration in metastatic melanoma between the responders to TIL therapy and the nonresponders; however, there was no significant difference [159]. One possible reason may be that the infiltrations of inflammatory and lymphocytic cells were not randomly distributed in the tumor. For example, they found a significant increase of FoxP3⁺ CD4 Treg cells in the intratumoral areas compared with peritumoral areas [159] and illustrated the selective accumulation of T cells in tumor. Galen et al. set up an immunoscore approach by immunohistochemical technology to analyze the location, density, and function of different immune cell types. It is important and useful for routine clinical use for classification of cancer, identification of the prognostic factors for disease-free survival (DFS) and overall survival (OS), and the potential targets for immunotherapy [160–162].

Recent studies suggested the immunosuppressive mechanisms in tumor microenvironment that inhibit T-cell activation [163] might also explain the reason for resistance of tumor immunotherapies. Gajewski's group found that the inhibitory pathways might have a negative feedback loop. Their results show that CD8⁺ T-cell-infiltrated metastatic melanomas had higher expression of inhibitory factors, indoleamine-2,3-dioxygenase (IDO), PD-L1/B7-H1, and FoxP3⁺ regulatory T cells (Tregs), and therefore are candidates for T-cell function inhibitors at the tumor site [20].

2.6 Conclusions: Where Is It All Heading?

The immune system has a remarkable capacity to recognize and control cancer growth and eradicate cancer cells through a variety of innate and adaptive effector mechanisms both at early stages and later stages either through *de novo* mechanisms or by clinical interventions that enhance immune responses through immunotherapy, as described in this chapter.

Much progress has occurred in our molecular understanding of cancer and how it interacts with the immune system and what key immune system drivers or blockers in melanoma can be harnessed to treat early- and late-stage disease. A key concept that has become apparent is that we need to view each tumor differently as an independent entity and that the tumor microenvironment needs to be viewed in context of the whole organism (cancer patient) where it forms a new ecosystem within the host as a “wound that never heals” or as “wounds in different parts of the body that have great trouble healing.” It has become apparent that tumors can regulate the immune response in the host both at short and long distances (e.g., exosomes can travel long distances through the circulation as can some dislodged tumor cells). For example, metastatic melanoma cells can induce the differentiation of suppressive MDSCs from monocytes through prostaglandins and COX-2 as well as through the release of

TGF- β . These MDSCs can express chemokine receptors (e.g., CCR7) that promote their homing not only to local but also in some cases distal lymphoid organs (lymph nodes and spleen) that do not harbor any tumor cells at all. At these sites, these MDSCs can set up an environment of systemic immunosuppression, especially in context of tumor-derived antigens that can be carried to these sites by these suppressive myeloid cells or, as recently demonstrated, through the uptake of tumor-derived exosomes that can traffic to these sites [122–124]. Tumor-derived exosomes can not only contain tumor biomarkers and tumor antigens but also contain factors, such as micro-RNA and other non-coding RNA, and immunosuppressive proteins that can be internalized by MDSCs at these local and distal lymphoid organ sites inducing T-cell tolerance, T-regulatory cell activation, and the inhibition of effector T-cell responses through the uptake of exosome-derived mediators directly into responding T cells [122–124]. Thus, tumors can elicit long-distance control over immune responses. This necessitates us to study the cancer patient as a whole ecosystem linking biomarkers of the immune response and tumor progression and metastasis and control in a systems biology approach looking at both systemic and tumor-localized proteomic and genomic factors regulating these processes.

Another area in which key concepts are rapidly changing is how we view the presence or detection of immunosuppressive pathways, especially at the tumor site. In the Introduction, we introduced the concept that the immune system is just beginning to recognize cancer as a problem, especially now in this new human age where life spans are increasing in length. Up until recently human evolution is needed to deal with the “enemy” of infectious disease to ensure survival through reproductive age. This protective process also needed coevolution of a counteractive process to limit or balance highly activated or powerful immune responses by invoking negative feedback processes that inhibit adaptive inflammatory responses to prevent immunopathology and autoimmunity. We are now also beginning that in fact this negative feedback or suppression of overly strong immune responses is also the key problem preventing effective tumor control during *de novo* immune responses or during responses elicited during immunotherapy. Thus, the focus of immunotherapy has shifted recently into both activating stronger antitumor responses (e.g., through cancer vaccines and proinflammatory cytokines like IL-2) and preventing this feedback inhibition through immunomodulators that inhibit signaling pathways, such as CTLA-4, PD-1, amino acid deprivation, and suppressive cytokines (e.g., TGF- β). The latter has been collectively called immune “checkpoints,” with “checkpoint blockade” using drugs and antibodies used to overcome these negative factors or checkpoints. Thus, an effective immune response against cancer must balance these two processes and future work will need to identify the situations (specific types of cancer and cancer stages and locations in the body) and how we fine-tune this balance between regulating these two processes. In addition, immune-based biomarkers in different patients will be critical to determine when and how we should use immune-activating or checkpoint blockade or both in specific circumstances. These biomarkers will identify patients with an active *de novo* antitumor response, using antitumor immune response measurements both in the blood and the tumor microenvironment (e.g., tumor biopsies) or those where a *de novo*

response is weak or missing. Therapies will need to manipulate the immune response based on these biomarkers, where in the former case, more activation and/or check-point blockade may be applied and, in the latter case, a stronger immune-activating regimen (both against the tumor and enhancing overall “immunocompetence”) may be needed. This new reality has also beginning to transform the way we interpret the detection of immunosuppressive biomarkers especially at the tumor site. Recent biomarker data now suggests that it is not the tumor cells that intrinsically turn on immunosuppressive pathways at the tumor site, but rather the immune system itself is orchestrating this. Thus, biomarkers of immunosuppression are being reinterpreted as being positive markers and not negative markers indicative of a stronger innate and adaptive immune response rather than a suppressed response. This contrasting view to our previously held beliefs will rewrite how we study antitumor immunity and how we apply immunotherapy. As shown in Fig. 2.5, this new conceptual framework can allow us to stratify patients toward different types or groups of thera-

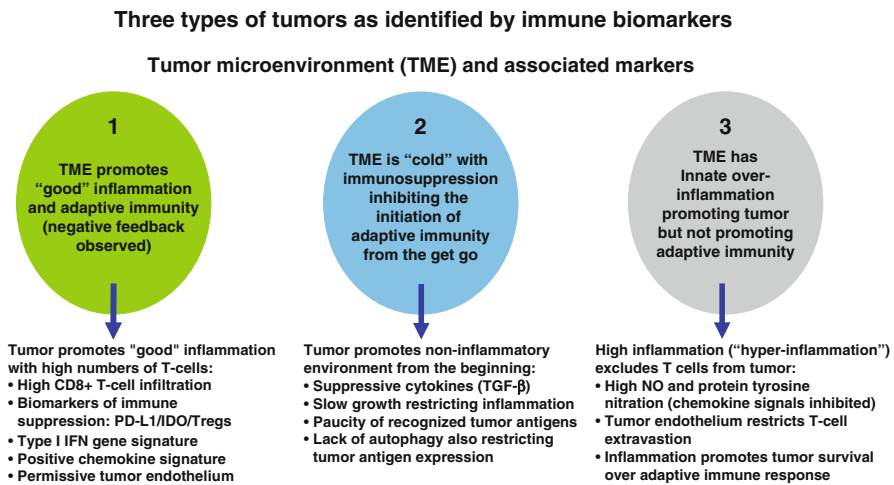


Fig. 2.5 New conceptual framework can allow us to stratify patients toward different types or groups of therapies based on the characteristics of their tumor-localized immune response. The goldilocks principle of tumors based on immune biomarkers: (1) “good” inflammation where a balanced set of innate immune factors and adaptive immune factors coupled with chemokine expression and DC activation leads to a high degree of T-cell infiltration versus too much infiltration by other myeloid cells such as macrophages and MDSCs; (2) non-inflammatory or “cold” tumor microenvironment that limits inflammation and infiltration by adaptive immune cells through cancer cell-derived powerful, intrinsic immunosuppression, such as high TGF-β release; and (3) “hyper-inflammation” in which the local inflammatory response is directed away from a more balance set of factors facilitating the migration of T cells and other adaptive immune cells into the tumor microenvironment and their survival and effector function toward an *over-production* of innate cytokines such as IL-1α and IL-1β, TNF-α, IL-6, IL-12, VEGF, endothelins, and Toll-like receptor agonists that not only feedback inhibit T cells but also drive strong NFκB activation in tumor cells and tumor cell survival and further secretion of these “negative” factors in a positive feedback loop. This latter tumor microenvironment may be exemplified by high peroxynitrite and protein nitration that can inhibit the function of key proteins such as chemokines and chemokine receptors or the activation of endothelial cells needed to drive T cells into the tumor

pies based on the characteristics of their tumor-localized immune response or markers in context to their systemic immune system or state of their overall immune system or “immune health” that will have another set of measured values. This will be the new reality for cancer care.

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