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Review

Tumor vaccines in 2010: Need for integration

David Koos [a,*], Steven F. Josephs [a,1], Doru T Alexandrescu [b,1], Ray Chun-Fai Chan [c], Famela Ramos [a], Vladimir Bogin [d], Vincent Gammill [e], Constantin A. Dasanu [f], Rosalia De Necochea-Campion [g], Neil H. Riordan [h], Ewa Carrier [g]

[a] Entest BioMedical, San Diego, CA, USA
[b] Georgetown Dermatology, Washington DC, USA
[c] Bio-Quant, San Diego, CA, USA
[d] Cromos Pharma Services, Longview, WA, USA
[e] Center for the Study of Natural Oncology, Del Mar, CA, USA
[f] Department of Hematology and Medical Oncology, St. Francis Hospital and Medical Center, Hartford, CT, USA
[g] Moores Cancer Center, University of California San Diego, CA, USA
[h] Medistem Inc, San Diego, CA, USA

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ABSTRACT

Induction of tumor-specific immunity is an attractive approach to cancer therapy, however to date every major pivotal trial has resulted in failure. While the phenomena of tumor-mediated immune suppression has been known for decades, only recently have specific molecular pathways been elucidated, and for the first time, rationale means of intervening and observing results of intervention have been developed. In this review we describe major advances in our understanding of tumor escape from immunological pressure and provide some possible therapeutic scenarios for enhancement of efficacy in future cancer vaccine trials.

1. Introduction

Despite theoretical promise, the area of cancer vaccines has been characterized by numerous set-backs. One of the tumor types most often used for immunotherapeutic investigations is melanoma, due to inherent immunogenicity and incidence of spontaneous regression [1]. Tumor antigens such as gp100 and tyrosinase [2,3] were originally identified in melanoma, as well as the cancer testis (CT) family of antigens that are expressed in numerous cancers, the first one being MAGE-1 [4]. Despite the wealth of knowledge surrounding melanoma immunology, clinical success has been non-existent. For example, Canvaxin, an irradiated whole cell vaccine, showed positive results in non-randomized studies [5], but in Phase III trials actually was associated with a trend towards decreased survival as compared to BCG [6]. Melacine, a whole tumor cell lysate also demonstrated positive results in Phase II studies [7], but in Phase III failed to provide a survival advantage over interferon in patients with resected melanoma [8]. This trend of failures in Phase III can be seen in other types of cancer vaccine trials. In prostate cancer, G-VAX, a GM-CSF secreting allogeneic cell based vaccine resulted in abnormally high patient deaths in Phase III, caused halting of clinical trials [9]. Antigenic's OncoPhage autologous heat-shock protein (HSP) vaccine failed to achieve US and EMEA registration for renal cancer [10], although in a Russian study achieved regulatory approval. Despite failure at a clinical level, many of these studies were able to generate cytotoxic T lymphocytes, or elicit other indications of immune response activation.

Over the last several years, molecular identification of the "battle" between the immune system and cancer has been occurring at an accelerating rate. The concept is developing that while one aspect of immunity may be stimulated by approaches such as vaccines, other arms of the immune system may be paralyzed or inhibited directly by the tumor, by tumor secreted soluble factors, or indirectly through tumor-induced regulatory immune cells. In this paper we overview some of the mechanisms by which tumors actively manipulate the immune response in order to provide a foundation for possible therapeutic means of immunological "derepressing".

2. Immune recognition of cancer

The importance of immunity in control of various tumors has been suggested by epidemiological studies showing correlations between natural and induced conditions associated with immune suppression and higher risk of malignancy. It is well-known that

* Corresponding author. Address: 4700 Spring Street, Suite 203, La Mesa, CA 91942, USA. Fax: +1 6193302328.
[1] These authors contributed equally to the manuscript.

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organ transplant patients under chronic immune suppression face a particularly exaggerated risk of neoplasia compared to the generation population [11]. One of several examples is a recent population-based cohort study which showed a significant elevation of head and neck squamous cell carcinoma (HNSCC) incidence was associated with long term immunodeficiency not only as a result of organ transplantation, but also because of hematological malignancy and HIV infection [12]. In some situations when cancer patients receiving immune suppression for unrelated conditions are temporarily withdrawn from the immune suppression, tumor remission has been reported [13].

A further suggestion that lack of immunity may be associated with tumor development comes from reports showing correlation between incidence of neoplasia and specific alleles of genes associated with innate immune system activation, such as MICA [14]. Animal studies support the role of immune system in control of cancer in general, as demonstrated by highly enhanced incidence of spontaneous carcinogenesis in the mice lacking cancer-detecting immune recognition receptors such as NKG2D [15]. In the case of HNSCC, administration of chemical carcinogens to p53 null animals in an immune competent background results in long tumor latency and localization, whereas accelerated growth curves and distant metastasis are observed when the same model is placed on a nude background which lacks T cells [16].

Immune surveillance implies existence of cancer-specific markers that are recognized immunologically. Such markers may be protein epitopes presented on MHC molecules that activate conventional T cells, or may be products of stress or DNA damage that induce activation of innate immune systems such as NK cells.

The majority of work in identification of T cell epitopes has been performed in melanoma, which inherently is a more immunogenic tumor as compared to other neoplasms. However, lessons learned from melanoma have been applied to squamous cell carcinoma. For example, numerous CT antigens originally identified in melanoma are also found in SCC, although consistency of expression is variable [17]. A study of 47 patients with oral SCC revealed expression of MAGE-A in 55% of tumors, with expression levels related to tumor undifferentiated status and invasiveness in lymph nodes [18]. MAGE-A3/6 and NY-ESO-1 have been found in some patients with SCC of the lung [19]. Epitopes from the CT antigens TTK protein kinase (TTK), lymphocyte antigen 6 complex locus K (LY6 K), and insulin-like growth factor (IGF)-II mRNA binding protein 3 (IMP-3) have been demonstrated to elicit CD8 responses in 20–70% of HNSCC patients tested [20]. Interestingly, vaccination with these peptides resulted in clinical responses in 5/10 HNSCC patients [21].

Other tumor-associated antigens of relevance to SCC include hyaluronic acid mediated motility (RHAMM) and carboanhydrase IX (G250/CAIX), which were found in 73% and 80%, respectively of HNSCC biopsy samples [22]. It appears that both of these antigens are immunogenic in vivo since in 4/8 HLA-A2+ patients, 0.06–0.13% of CD8+ effector T cells recognized tetramers for RHAMM or G250 and secreted IFN-γ and granzyme B in ELISPOT assays. The tumor suppressor protein p53 is overexpressed in its wild-type form or mutants in HNSCC. Investigators have identified T cell epitopes of p53 that stimulate patient CD8 cytotoxic lymphocytes in vitro [23]. A recent study demonstrated that HLA-DP5-restricted T cells responsing to wild-type p53 derived epitopes appear to have a Th1 profile in patients with early tumors, and as tumors advance a switch to a Th2 is observed [24]. Supporting the possible relevance of p53 as a dominant tumor antigen are studies in which correlations have been made between presence of T cells specific to p53 and tumor infiltrating lymphocytes in patients with cutaneous SCC [25]. Epitopes of EGFR have been identified to be immunogenic, with elevations of CD8 cells in circulation of patients with HNSCC that are capable of recognizing EGFR853-861 [26]. The importance of EGFR as a target in HNSCC is exemplified by FDA approval of Cetuximab, an antibody against EGFR, for use in combination with radiation therapy for treating HNSCC, or as a single agent in patients who have had prior platinum-based therapy [27].

In addition to activators of adaptive immunity, HNSCC cells have been found to express activators of NK cells that mediate effects through the NKG2D receptor. The NKG2D ligands MHC class I-related chain molecules A (MICA) and UL16-binding proteins (ULBPs) have been found to be overexpressed in the human laryngeal carcinoma cell line Hep-2 and primary HNSCC as compared to non-tumor tissues of vocal cords polyps [28]. However, other studies have demonstrated significant variability of expression [29].

Therefore, it appears that there is some degree of immune control of spontaneous cancers, and there is some molecular basis for which immune cells may specifically kill neoplastic cells as compared to controls. The next question is whether in vivo there is infiltration of immune cells and what role do they play in the clinical prognosis.

3. A look in the tumor: TILs

Tumor infiltrating lymphocytes (TILs) are believed to be a manifestation of immunological attack on tumors. Early investigation of TILs led to establishment of principles of tumor immunology such as recognition of common melanoma antigens, HLA restriction of response, and the relationship between infiltration and positive prognosis [30,31]. These observations gave rise to the concept that if one could increase the number of tumor-recognizing lymphocytes in the patient, outcome could be improved. Although costly, the procedure of physically extracting TILs, expanding in vitro and subsequent re-infusion has yielded some clinical results, however significant impact on patient survival was not achieved [32,33]. With the discovery of advanced cell sorting and characterization procedures, it became apparent that some of the concepts were oversimplified and that TILs are actually a heterogenous population with both tumor inhibitory and stimulatory functions [34,35].

Several correlations have been made between TIL and favorable prognosis, however, this depends on characteristics of the tumor. For example, Distel et al. reported that in 62 HNSCC patients classified as "low-risk" based on early disease and treated with surgery followed by external radiotherapy, higher numbers of intraepithelial CD8 + TIL (>66.6 per thousand) was associated with disease survival of 95% vs 52% ($p = 0.005$) [36]. A study of 84 patients with newly-diagnosed HNSCC demonstrated positive relation between presence of activated CD4 T cells and survival using CD69 expression to define activation [37]. Presence of activated CD8 TILs based on IFN-γ production and proliferative potential also correlated with improved survival in esophageal carcinomas both of squamous or adenocarcinoma types [38]. In Stage 1B cervical SCC a relationship between number of TILs expressing both CD4 and CD8 and cancer free survival was observed when comparing biopsy samples from 20 patients that relapsed with 20 that did not [39]. The same group also reported association between low infiltrating CD3 cells and relapse in a study of 102 patients with Stages IB and IIA cervical SCC [40]. Interestingly in anal SCC presence of granzyme B+ TILs actually was a negative factor in prognosis [35]. In the case of melanoma, a study comparing 15 patients with poor outcome to 20 patients with favorable outcome found a correlation between survival and presence of granzyme B positive CD4 TIL [41]. A larger study of 875 melanoma patients found absence of TIL correlated with metastasis [42]. Correlations between TIL and favorable prognosis has also been made in ovarian [43,44], lung [45], breast [46], kidney [47], and colorectal cancer [48].

Thus generally speaking, in several types of tumors patient prognosis can be correlated to some extent with number of TILs. Studies examining activation state, cytokine status, and cellular subsets are anticipated to provide more precise prognostic information. However, it must be noted that other infiltrating cells may have negative effects. For example, macrophages, although known to possess spontaneous tumor cytotoxicity [49], and are involved in antibody dependent cellular cytotoxicity [50], may actually be tumor-promoting. In a 56 patient study with esophageal SCC tissue obtained after curative surgery, angiogenic index was associated with tumor infiltrating macrophages (TAMs) as detected by CD68 expression. Supporting a possible negative prognostic implications was that 5 year survival was lower in patients with higher TAMs [51]. Other tumor types such as those of breast, lung and renal origin have been associated with increased invasiveness when high levels of TAMs were detected. Besides angiogenic mechanisms, TAMs may be causing degradation of the TCR-zeta chain, an association that was demonstrated in a study of 137 esophageal SCC patients. The study also observed lower survival time associated with increased TAMs [52].

The fact that tumor-specific antigens exist, that they are capable of eliciting an immune response, and that in some situations immune response correlates with improvement suggests a foundation for pursuing means of stimulating immunity towards tumors. Unfortunately, attempts to do this have largely been unsuccessful. One of the possible explanations for this may be systemic alteration/suppression of the T cell compartment by the tumor or cells recruited by the tumor.

4. Passive suppression: T cells are systemically dysfunctional in cancer?

It has been known for decades that T cells in the TIL have some reduction in function as compared to circulating T cells, presumably as a result of the tumor elaborated immune suppressive factors [53]. Before describing specific mechanisms of immune suppression, we will first discuss some of the systemic immunological manifestations observed in tumor patients.

A simple method of testing systemic immunity is ex vivo stimulation of lymphocytes with non-specific mitogens such as phytohemagglutanin. These agents crosslink glycosylated receptors on T cells and cause proliferation independent of TCR specificity, thus assay measures general T cell proliferative and cytokine-producing activity. In several cancers a correlation between proliferative response to PHA and survival has been noted. Marana et al. described a relation between response to treatment, survival rates, and PHA-induced proliferative response in cervical cancer patients treated with cisplatin and bleomycin [54]. In a subsequent study, pre-surgery PHA responses were associated with improved survival in breast cancer patients [55]. This was also found in a study of 158 breast cancer patients that were operable and 52 that had metastatic disease in which length of disease free survival correlated with immune competence [56]. Suppressed PHA response in HNSCC patients as compared to healthy controls was also reported [57].

While PHA responsiveness provides a snapshot of overall T cell activity, it appears that specific subsets of immune cells are selectively targeted by the tumor. In a recent study of forty untreated HNSCC patients an inverse relationship between tumor infiltrating CD4 T cells and tumor advancement was noted in biopsy samples. This also correlated with reduced PHA proliferative responses in vitro peripheral PBMC [58]. In order to investigate the proliferative response defects in more detail, separation of CD4 and CD8 cells was performed using magnetic separation. Ex vivo expansion of CD4 but not CD8 cells was markedly inhibited in patients as compared to healthy controls [59]. To illustrate the importance of culture conditions and soluble factors generated by the tumor, in a different study, culture of HNSCC patient PBMC (from 61 patients) in autologous serum led to actually an increased proliferative response as compared to control (46 patients) [60]. The investigators found no difference in IL-2, IL-4, or IFN-γ between the groups. A subsequent study by the same group using a different culture system reported correlation between high lymphocyte proliferative response to mitogen and survival. Importantly, the predictive value was also retained when other factors were included in the survival analysis such as values of major serum proteins, hormones and smoking and alcohol history [61].

One explanation for the reduction in T cell activity is a phenomena associated with numerous inflammatory states in which the zeta chain of the T cell receptor is under expressed or cleaved. Since the zeta chain contains three immunotyrosine activating motifs, its integrity is critical for T cell activation [62]. Deficient zeta chain has been observed in conditions as diverse as rheumatoid arthritis [62], systemic lupus erythematosus [63], and hemodialysis patients [64] that all have an underlying inflammatory condition. One of the first observations of zeta chain downregulation was by Zea et al. who examined 44 patients with malignant melanoma. They showed that cells from patients with diminished TCR-zeta chain expression had lower IL-2 and IFN-γ compared with healthy subjects and melanoma patients with normal TCR-zeta chain status. Survival of melanoma patients with low TCR-zeta chain expression was significantly shorter than that of patients with normal TCR-zeta chain expression [65].

Original thoughts regarding zeta chain cleavage centered around oxidative stress or Fas-induced activation of caspase-3, which cleaves a DEVD domain found on the zeta chain [66–69]. Mediators of inflammation such as chronic TNF-alpha have also been demonstrated to downregulated zeta chain on T cells [70]. More recent studies have suggested that "TCRzeta(dim)" T cells are antigen-experienced cells refractory to TCR-induced proliferation that share characteristics of differentiated effector T cells but use accessory pathways for transducing signals for inflammatory cytokine gene expression and cell contact-dependent pathways to activate monocytes [71].

5. Active suppression: T regulatory cells

Control of autoreactivity has classically been ascribed to thymic deletion of self-reactive T cells. An alternate mechanism is the central and peripheral generation of a type of T cell capable of actively suppressing other T cell responses termed "T regulatory" (Treg) cells. Importance of Treg in controlling immunity is demonstrated in experiments showing their deletion leads to systemic autoimmunity, as well as exacerbation of ongoing immune responses to viral or bacterial agents [72,73].

Schaefer et al. found elevated numbers of CD4 + CD25+, FoxP3+, GITRL + Treg in patients with head and neck SCC both with active disease and post treatment that had no evidence of disease in comparison to healthy controls [74]. An increase in apoptotic (Annexin V+) and pre-apoptotic CD4 T cells was also observed in patients as compared to controls, which correlated with decreased expression of the TCR-zeta chain. Correlation between increased Treg and decreased cytokine-producing (IFN-γ and IL-4) CD8 cells was reported by an independent group in a similar patient population as compared to healthy controls [75]. In a 38 patient study local biopsy evaluation of anal squamous cell carcinomas revealed no association between Treg infiltration, as judged by staining for FoxP3. Perhaps paradoxically, shorter patient survival was associated with increased numbers of Granzyme B positive T cells infiltrating the tumor [35]. It may, however, be possible that the

Granzyme B staining cells were Treg not expressing FoxP3, since expression of Granzyme B as a Treg marker was not widely appreciated at the time of publication [76].

Mechanistically, the generation of Treg by SCC has been replicated in an in vitro culture system comprised of irradiated SCC cell line, immature dendritic cells (iDC), CD4(+)CD25(−)T cells, IL-2 (10 IU/ml), IL-10 (20 IU/ml), IL-15 (20 IU/ml). The authors found that these conditions, which mimic the tumor microenvironment, were capable of expanding a Treg subset called Tr1, which had the phenotype of CD3(+)CD4(+)CD25(−)IL2Rbeta(+)IL2Rgamma(+) FoxP3(+)CTLA-4(+) and mediated suppression by secretion of IL-10 in a contact-independent manner [77]. These findings have been replicated in another study showing that CD4 cells isolated from patients have a higher tendency to acquire the Tr1 phenotype as compared to cells from healthy volunteers [78]. Similar results have been reported using tumor ascites fluid or culture supernatant for squamous cell carcinoma or adenocarcinomas, in which the culture system supported acquisition of regulatory activity from CD4+ cells [79]. Possible importance of IL-10 in mediating suppression of T cells was suggested in early studies in which high concentrations of this cytokine were found in patient sera, and neutralization led to derepression of lymphocyte proliferation [80].

It remains interesting why the cells identified did not express CD25 as do conventional Treg. A similar contact-independent IL-10 secreting Treg cell lacking CD25 was observed in patients with head and neck SCC as tumor infiltrating lymphocytes [81]. The same group assessed levels of circulating Treg, based on CD4+, CD25+ expression, in 35 head and neck SCC patients as compared to 15 healthy controls. Patients had higher Treg numbers (5 ± 3%) as compared to controls, (2 ± 1.5%), as well as enhanced suppressive activity (78 ± 7%) vs (12 ± 4%) [82]. Indeed it may be the case that a CD25 negative suppressive population exists that is localized to the intratumoral environment. Alternatively, it may be that these cells acquire CD25 in the periphery. An independent group reported that although SCC patients have elevated CD4 + CD25 + Treg levels in the periphery, about 25% of the CD4 + CD25− cells express FoxP3 and display IL-10 induction ability [83].

While these data suggest Treg may play a role in immune suppression associated with head and neck SCC, a study by another group actually demonstrated positive correlation between CD4 + CD25 + TIL and overall survival [84]. The possibility that the CD4 + CD25+ cells detected in this study were activated T cells cannot be excluded. Techniques such as double chromogenic staining for FoxP3 and CD4 may provide more useful methods of analysis [85]. Despite this, a larger, 112 patient, study demonstrated association between peripheral blood Treg numbers and tumor recurrence [86].

Other tumors have also been reported to be associated with increased Treg numbers both systemically, and intratumorally. This has been the case for melanoma [87], kidney [88], liver [89–91], colon [92], and brain cancers [93].

6. Tolerogenic dendritic cells as generators of Treg and immune deviation

Broadly speaking, the argument has been made that tolerance is controlled to some extent by immature dendritic cells presenting self antigen in absence of costimulation/presence of co-inhibitors, which leads to generation of Treg cells and anergic T cells [94]. This was demonstrated in several systems, for example, in a classical experiment Mahnke et al. targeted the antigen ovalbumin to immature dendritic cells by conjugation to anti-DEC-205 antibodies. It was observed that antigen-specific Treg were generated, which was dependent on presentation by immature dendritic cells [95]. In vivo relevance of Treg generated by targeting antigen to steady state dendritic cells can be seen in studies where DEC-205 targeting of antigen prevented autoimmune diabetes in a transgenic model system via FoxP3 expressing Treg [96]. Min's group reported on a "tolerogenic vaccine" created by ex vivo generation of immature DC treated with a chemical IKK inhibitor, and pulsed with collagen II, that was able to prevent arthritis in a mouse model [97]. Similar tolerogenic uses of immature DC have been reported in diverse conditions such as transplantation [98], anti-Factor VIII immunity [99], autoimmune myocarditis, experimental autoimmune mysthenia gravis [100], and collagen induced arthritis [101]. The possibility that tumors may be generating immature DC to protect themselves from T cell attack and/or generate Treg was suggested in studies showing tumor secreted VEGF would arrest DC maturation in vitro [102]. Mechanistically it was demonstrated that VEGF blocks NF-kB activity in DC, which is a critical maturation-inducing factor [103]. Given that VEGF is a primary cytokine in tumor angiogenesis, the possibility of inhibited DC maturation being a mechanism of immune escape is attractive. Angiogenesis seems to be associated with various cells of the myeloid lineage. The myeloid suppressor cell, which will be described below, has been demonstrated stimulate angiogenesis directly, and through production of MMP-9 and VEGF [104].

In HNSCC a population of myeloid suppressor cells was described in a series of publications by Rita Young's group. These cells, which express the hematopoietic stem cell marker CD34, were originally identified as the source of intra-tumor GM-CSF detected from primary patient samples [105]. Suggesting a possible immune inhibitory role for these cells were data that their depletion results in upregulated ability of lymphocytes within the tumor to generate IL-2, which was lost upon re-introduction of these cells into culture. Clinical relevance of these myeloid suppressor cells was supported by a study of 20 HNSCC patients whose tumors were resected and relapsed, compared to 17 patients that had disease free survival for the 2-year observation period. Tumors of patients relapsed produced almost 4-fold higher levels of GM-CSF and had approximately 2.5-fold the number of CD34+ cells as compared to patients that were free of disease [106]. Mechanistic study of these cells revealed suppression of T cell activity could be abolished treatment with antibodies to TGF-b, and that inhibitory activity was lost upon their differentiation with agents such as IFN-γ and TNF-alpha [107]. Given that immature DC mediate Treg generation through TGF-b [94], and that immature DC lose inhibitory activity upon maturation with agents such as IFN-γ and TNF-alpha, the possible relationship with myeloid suppressor cells was considered [108]. In fact, a recent study suggested the possibility of vivo differentiation of myeloid suppressor cells. Newly-diagnosed HNSCC patients were treated with Vitamin D3 for three weeks before surgical excision of the tumor. Observations of significant reduction in numbers of intratumoral CD34 cells and augmented numbers of dendritic cells were reported [109]. Other interventions for induction of myeloid suppressor cell differentiation into DC/reversing immune suppressive potential have demonstrated some promise including 5-azacytidine [110], sunitinib [111], PDE-5 inhibitors [112], and inhibitors of stem cell factor or its receptor c-kit [113]. Of these, 5-azaycytidine, sunitinib various PDE-5 inhibitors are already part of clinical practice. In the case of sunitinib, clinical evidence of derepression of T cell responses after therapy has been reported [114], effects being mediated, in part, by suppression of STAT3 activity [115]. Myeloid suppressor cells have been described in numerous other conditions of neoplasia, in which GM-CSF has been reported to be a major factor in their generation [116,117]. In addition to TGF-beta, suppression by myeloid suppressor cells seems to be mediated by PGE-2 [118], expression of arginase, which generates immune suppressive polyamines [104], and depletion of cystine and cysteine [104] (amino acids needed for T cell activation).

Thus while it is still not completely clear how upstream in the differentiation pathway myeloid suppressor cells are as compared to immature dendritic cells, there is evidence that both cell populations mediate generation of Treg cells [119]. In the case of HNSCC at least one paper supports in situ generation of Treg by immature antigen presenting cells, specifically, a study comparing SCC with Actinic Keratosis demonstrated that increased Treg cell numbers were associated with local DC, in SCC [120]. Others have made correlations between myeloid suppressor cell numbers and Treg [121–123].

7. Treatment concepts

In addition to providing an antigen-specific stimuli for augmentation of T cell immunity, combination immunotherapies would have to take into consideration three main areas: (a) providing innate stimulators of immunity; (b) inhibiting systemic inflammation/oxidative stress that is present at a baseline-level but would be augmented during systemic immune activation; and (c) inhibition of immune inhibitors. We will discuss some possible approaches and rationale to these, as well as methods of monitoring success before clinical progression/regression occurs.

7.1. Non-antigen specific approaches

Historically, it appears that of the few immunotherapies used, the non-specific activators have been the ones to achieve most repeatable success on cancer. The first such approach was the work of William Coley from Sloan Kettering more than 100 years ago, who obtained treatment success of soft tissue sarcoma patients with immune stimulatory bacterial preparations [124]. In 1995 the FDA approved interferon alpha for treatment of melanoma [126–128], and interleukin-2 in 1998 for melanoma [129], and subsequently for renal cell carcinoma. Although the rationale for development of both of these agents has been immunologically-based, and correlation between response and various immune parameters have been made, both agents also exert immunologically-independent actions that may be responsible, at least in part of therapeutic effects. Unfortunately, toxicity is associated with both immune modulators, and the responding population is relatively small.

With the advent of molecular biology, many of the stimulators of innate immunity have been identified and testing in a variety of preclinical and clinical systems. Members of the toll like receptor (TLR) agonist family such as Poly-IC (TLR-3), CpG motifs (TLR-9), imiquimod (TLR-7/8) have been used in a variety of oncological conditions with mixed responses [130]. To date, none have achieved regulatory approval with the exception of imiquimod for cutaneous basal cell carcinoma. The strength of using innate activators of immune resides in the fact that knowledge of tumor antigens is not necessary. As can be seen in our discussion of HNSCC antigens, tumors despite having the same macroscopic and histological properties often express numerous different antigens. Additionally, tumor downregulation of MHC or antigen processing machinery such as TAP-1 may block ability of adaptive immunity to mediate effects. An interesting way to overcome this is through administration of agents that induce re-expression of antigen processing molecular machinery through epigenetic means. One such example is the clinically-used histone deacetylase inhibitor valproic acid, which has been demonstrated to evoke melanoma cells to re-express molecules such as TAP and MHC 1, while functionally inducing antigen-presentation ability [131].

Unfortunately, non-specific activation of innate immune may also be associated with downside. The first is the obvious one of toxicity. Beginning from the work of Coley, the limiting factor to activation of systemic immunity has been fever and symptoms associated with systemic cytokine activity. Interferon alpha, while being markedly more effective at high doses for increasing clonal proliferation of anti-tumor T cells [132], is limited by toxicities including transient ischemic episodes, fatigue, and depression [133]. Interleukin-2 associated toxicities are even more severe, with lethality occurring in some cases [134]. Biologically these are caused by, *inter alia*, vascular leak syndrome [135–137], systemic activation of complement [138,139], and rise in numerous inflammatory parameters such as TNF-alpha [140,141]. Interesting approaches may be considered in reducing toxicity, for example, administration of histamine to "precondition" the system to oxidative stress has demonstrated benefit [142]. An additional intervention that may be considered is co-administration of intravenous vitamin C. It has been previously reported that IL-2 therapy causes a severe drop in endogenous ascorbic acid levels to undetectable range [143,144]. Additionally, clinical scurvy has been reported in patients receiving IL-2 therapy [145]. Given the essential role of ascorbic acid in maintaining endothelial health [146–148], combined with direct anticancer effects of ascorbic acid at high concentrations achievable only by intravenous administration [149–153], a combination study may be useful to be performed in preclinical settings.

Some tumors express TLRs, which have shown that TLR activation on tumors actually increases tumor-immune evasion mechanisms [154]. However, these results are controversial in light of another study demonstrating direct tumor inhibition by TLR ligation [155]. Additionally, myeloid suppressor cell activation is actually increased by TLR4 ligands [156]. Given the limited responses seen with innate activators of immunity, the question becomes whether higher intensity of administration is needed, whether the right innate activators were used, or whether innate immune activation needs to be performed in combination with other approaches.

One method of therapeutically using activators of innate immunity is by combination with therapies that induce release of tumor antigens. For example, procedures that induce in vivo tumor death such as suicide gene therapy [157], cryosurgery [158,159], radiofrequency ablation [160], and radiotherapy [161] have been associated with induction of tumor immunity. It is known that in the basal state dendritic cells present antigens in a tolerogenic manner [96]. Tumor associated dendritic cells have an increased tolerogenic activity due to tumor derived VEGF and other soluble factors [103]. Conceptually modulation of the local antigen presenting environment through use of innate immune activators subsequent to induction of antigen release may be a promising method of overcoming lack of knowledge related to specific antigens. The importance of innate immune activation under correct parameters can be seen in the ability of certain "danger signals" to abrogate tolerance. It may be possible that by inducing local antigen release and activation of DC, a synergy in immune cell activation may occur. This would be analogous to situations in which TLR signaling is used to break tolerance. This can be seen in many conditions of tolerance ranging from pregnancy, in which administration of TLR4 agonists like LPS or TLR9 agonist in IL-10 deficient background leads to immunologically mediated abortion [162]. Breaking of tolerance has also been demonstrated in transplant models where administration of TLR4 ligands results in graft rejection [163]. Mechanistically, several of the TLR ligands inhibit tolerance through making T cells resistant to inhibitory effects of Treg cells [164,165],

As part of innate immune activation, various feedback inhibitory reactions occur. Upregulation of the immune suppressive enzyme indolamine 2,3 deoxygenase (IDO) occurs in the presence of immune activators associated with anticancer immunity such as interferon gamma [166], IL-2 [166], TLR agonism in context of

PGE-2 [167], and IL-12 [168]. The critical role of IDO in tolerance induction and maintenance can be seen in situations as diverse as pregnancy [169], autoimmune arthritis [170], and cancer [171], in which inhibition of the enzyme results in loss of tolerance or enhancement of immunity. To achieve maximum immune attack, one would need to take into consideration IDO and other inhibitory feedback loops that may be present. Wei-Ping Min's group was able to induce regression of melanoma in the B16 model by administration of antigen-pulsed DC with siRNA-mediated IDO inhibition either locally or systemically [172].

7.2. Oxidative stress

Another negative feedback inhibitor of immunity is oxidative stress. Activation of innate immunity, for example, macrophages with LPS, results in generation of free radicals that are capable of suppressing T cell responses. The concept of TCR-zeta chain cleavage was discussed above as being linked to oxidative stress. Specific experiments demonstrating this is that co-culture of activated macrophages leads to zeta chain degradation, or that zeta chain loss after incubation of neutrophils from cancer patients with healthy lymphocytes is abrogated by addition of catalase. In addition to immune activation causing oxidative stress, the tumor itself generates large amounts inherently. This compounds the fact that the tumor environment inherently induces oxidative stress. Chemokine production by tumors occurs in part due to hypoxia, which activates transcription factors such as HIF-1 [173]. Infiltration of tumors by macrophages is associated with inflammation, release of free radicals, and poor prognosis [174–176]. Mediators producted by macrophages such as NO, OH, and H_2O_2 have been directly demonstrated to cause cleavage of TCR-zeta chain [177,178]. Another important mechanism of oxidative stress mediated immune modulation is the selective resistance of Treg to oxidative stress [179]. Given the high level of tumor-associated oxidative stress, it may be possible to imagine that selective depletion of conventional T cells is occurring in the tumor environment.

Modulation of oxidative stress in the tumor environment may conceptually be performed by systemic administration of various antioxidants. For example, n-acetylcysteine is clinically used for acetaminophen poisoning, based on ability to scavenge hepatic-damaging free radicals. Administration of this compound in vitro has been demonstrated to inhibit tumor-associated reduction of TCR-zeta chain [180]. Another antioxidant that may be considered for reversion of systemic oxidative stress is intravenous ascorbic acid. Pilot clinical studies have demonstrated anticancer effects of this approach [181,182], although recent work suggests that tumor cytotoxicity was occurring due to generation of intratumoral free radical production [151].

7.3. Inhibition of immune inhibitors

The inhibition of immunity by natural suppressor cells of the myeloid lineage has been widely described. It appears that VEGF production is associated with maturation block of these cells [183]. Although the anti-VEGF antibody has been approved for renal, breast, colorectal, lung, and glioma in various combinations [184], work remains to be performed at optimizing effects of this drug. The possibility of concurrently inhibiting neovascularization with providing immune stimulation is enticing. Alternative methods of modulating angiogenesis may involve use of naturally derived compounds. For example, it is known that agents curcumin, an ingredient from turmeric has been demonstrated in in vitro studies to inhibit HUVEC proliferation, MMP activity, and in tube formation [185]. Studies in the U-97 human glioma xenograft model revealed potent inhibition of tumor growth, associated with reduction in angiogenesis [186]. Similar results have been reported in syngeneic bladder cancer models [187], and hepatocellular carcinoma models [187]. Another natural inhibitor of angiogenesis is epigallocatechin-3 gallate (egcg, from green tea) [188], which also seems to exert effects through inhibition of VEGF production [189]. It will be interesting to see if such natural inhibitors of VEGF may be used for restoration of immunity.

8. Conclusion

Although substantial progress has been made in understanding the tumor-immune interaction at a molecular and cellular level, clinical translation is only beginning to occur. Entry of agents such as antibodies to CTLA4, VEGF, and CD25 offer potential means of combating cancer immune suppression, however these are not routinely used in combination with antigen-specific and non-specific immune stimulators. Future immunotherapeutic research in the area of cancer should address multifactorial nature of the disease using combination protocols. We attempted to pull together several such ideas that are usually presented in a discordant manner, portray a framework for future immunotherapeutic studies.

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