Releasing the Brakes in Cancer

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Abstract

Cancer is a heterogeneous group of diseases where abnormal cell growth with potential to invade other body parts takes control of normal homeostasis and becomes fatal if not timely and rightly treated. There are more than 100 types of cancers characterized so far and many yet to be identified. World Health Organization estimates, that worldwide in 2012 there were 4 million new cancer cases and 8.2 million cancer related deaths. Amongst various treatment options available for cancer, immunotherapy offers an approach where the focus is on enhancing or even inducing an antitumor immune response. Induction or enhancement of anti-tumor immune response is a formidable challenge in cancer because tumor cells use multiple immune evasion strategies and avoid being detected or eliminated by immune cells. Immune checkpoints refer to a network of stimulatory or inhibitory signaling pathways in the immune system which are critical in maintaining self-tolerance, limiting tissue damage and modulating the quality of immune response. Substantial evidence indicates that up regulation of inhibitory signaling molecules (CTLA-4, PD-1) by tumor cells subvert activation of tumor antigen specific Teffector cells. Therefore, blockade of inhibitory signaling pathways may be one potential way of revitalizing an exhausted immune response in tumors. Using this approach, antibodies directed against CTLA-4 and PD-1 have been shown an acceptable therapeutic benefit in preclinical models and cancer patients. This review will discuss the important immune checkpoints that have been identified critical to suppress anti-tumor immunity and have been exploited as drug targets.

Keywords: Cancer stem cells; Anti-tumor; Lymphatic

Tumors develop as a result of uncontrolled cell growth, avoiding programmed cell death and often bypassing the signals generated to restrict cell division [1]. During this uncontrolled growth, cancer cells undergo profound cellular and molecular changes forming a complex niche known as the Tumor microenvironment (TME), which comprises of cells of tumor origin with genetic alterations and genetically unaltered non-malignant cells such as fibroblasts, endothelial cells (blood and lymphatic), mesenchymal cells and components of extracellular matrix [2]. It is now evident that the stromal structure is critical for tumor sustenance and it creates a pathway for infiltration of various immune cell types like natural killer cells (NK), macrophages, activated T-cells, tumor associated macrophages (TAM) and myeloid derived suppressor cells (MDSC). Since the formulation of "immune surveillance" hypothesis at the beginning of 20th century by Paul Ehrlich and later refined by Burnet and Thomas in 1950's, immune cells particularly lymphocytes and NK cells have been established as critical for detection and destruction of tumor cells [3].

According to the concept of cancer "immune editing", immune selection favors the emergence of tumor variants that have accumulated antigenic alterations sufficient for the evasion of immune surveillance mechanisms leading to tumor progression [4]. Even though the down regulation of MHC I on tumor cells invoke a robust NK cell activity leading to tumor cell lysis, T-cells are critical to control tumor cell expansion. A concerted interaction between both innate and adaptive immune system is important for elimination of cancer cells [5]. Appropriate cues from a prior innate immune response influence T cell differentiation i.e. innate signals from cells like classically activated macrophages and dendritic cells induce activation of cytotoxic T cells (CTLs) and T helper (TH1) cells which is beneficial to the host in eliminating tumor cells [6]. On contrary, signals generated from alternatively activated macrophages (AAM) or myeloid derived suppressor cells (MDSC) [7,8] promote tumor metastasis and progression **(Figure 1)**. Clinical manifestations of cancer may be visible once the immune response skews to promote tumor progression. Immunotherapy augments anti-tumor immune response by rewiring host immune response from tumor progression to tumor elimination **(Figure 1)**.

Regulation of T Cell Responses

Both CTL and Th1 cells are crucial for an effective anti-tumor immune response. Being important mediators of anti-tumor immunity, T cells are most favored targets for translational development of immunotherapeutic molecules. A variety of tumor specific (TS)
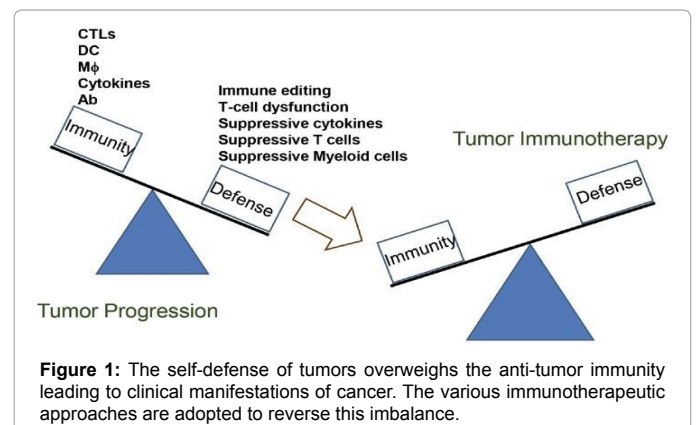

Figure 1: The self-defense of tumors overweighs the anti-tumor immunity leading to clinical manifestations of cancer. The various immunotherapeutic approaches are adopted to reverse this imbalance.

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or tumor associated (TA) antigens derived from oncogenic viruses (HPV, SV40), differentiation antigens (Tyrosinase, Carcinoembryonic antigens, Alpha-fetoprotein, prostate-specific antigen), epigenetically regulated antigens (cancer antigen-1, MAGE-antigens) and neoantigens allow T cells to distinguish between normal and transformed cell [9,10]. Induction of effector T cell response is sequential, antigen specific T cells are first primed in the secondary lymphoid organs through the interaction with antigen presenting cells (APC). APC particularly dendritic cells (DC) sample antigens from tumor cells and present antigens to CD4$^+$ T cells via the MHC class- II pathway or to CD8$^+$ T - cells via cross presentation or cross priming [11,12]. This antigen recognition in association with MHC is insufficient to effectively activate T cells; APC provide additional cosignals that regulate the breadth of T cell activation. These multiple cosignals can be induced by stimulatory (CD80 / CD86: CD28) and inhibitory molecules also known as "Immune checkpoints" [13,14]. The entire process of T cell activation and differentiation is finely regulated by a balance between multiple stimulatory or inhibitory receptors on T cells and their respective ligands present on APC (Table 1). APC also provide the additional costimulatory signals, which are mandatory for T cell priming. After the priming phase, several factors, including but not limited to defective T cell recruitment at tumor site, inactivation of effector functions of primed T cells or induction of T cell apoptosis contribute to the diminished response of antigen specific T cells at the site of tumor development ultimately causing reduced cancer elimination.

T cells	APCs / Tumor cells	Effect
Immunoglobulin family		
CD28	B7.1 / B7.2	+
ICOS	ICOS-L	+
CTLA-4	B7.1 / B7.2	-
PD-1	PDL-1 / PDL-2	-
?	B7-H3	-
?	B7-H4	-
BTLA	HVEM	-
LAG-3	MHC-II	-
CD160	HVEM	-
VISTA	?	-
TNF-R family		
CD4L / CD154	CD40	+
OX-40	OX-40L	+
4-1BB	4-1BBL	+
GITR	GITR-L	+
CD30	CD30L	+
HVEM	LIGHT	+
T cell immunoglobulin mucin family		
TIM-1	?	+
TIM-2	?	-
TIM-3	Galactin-9	-
Butyrophilin family		
BTN	BTNL	-

+ indicates stimulation and - indicates inhibition of T cell activation. ? Indicates receptor or ligand yet to be identified. ICOS: Inducible costimulator of T cells [67] ,CTLA-4: Cytotoxic T Lymphocyte Antigen 4 [68], PD-1: Programmed Death 1 [69], BTLA: B and T Lymphocyte Attenuator [70], LAG-3: Lymphocyte Activation Gene [71], VISTA: V -domain Ig Suppressor of T cell Activation [72], OX-40: CD134 [73], 4-IBB: CD137 [74], GITR: Glucocorticoid-Induced Tumornecrosis factor Receptor [75], HVEM: Herpes Virus Entry Mediator [76], TIM: T cell Immunoglubulin Mucin family [77,78], BTN: Butyrophilin [79]

Table 1: Costimulatory molecules and their corresponding ligands expressed on T cells and APCs / tumor cells.
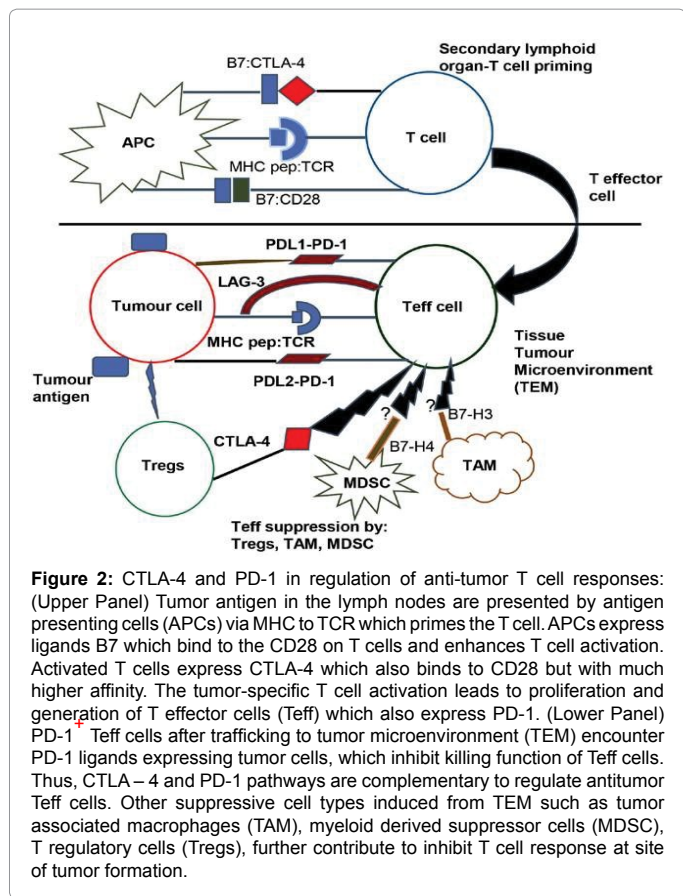
Of the various proteins that regulate T cell activation (Table 1), Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), Programmed Death-1 (PD-1), B7 family members B7-H3, B7-H4, T cell Immunoglobulin and Mucin domain-containing protein 3 (Tim-3), and Lymphocyte Activation Gene-3 (LAG-3) block costimulation and abrogate the response of activated T cells (Figure 2). Abnormal expression of either of these inhibitory checkpoint molecules is a predominant immune evasion mechanism in cancers, chronic infections and autoimmune diseases. In this review, we will discuss the important immune checkpoints that have been identified critical to suppress anti-tumor immunity and have been exploited as drug targets. We will also discuss other immune check points and their antagonists in preclinical development for various cancers.

CTLA-4 First Target Identified to Release Brakes

Amongst all the therapies that have been used to potentiate immune response against cancer, immune checkpoint blockade has shown most promising results and has been appropriately heralded as a major scientific breakthrough in translational research. CTLA-4 was the first inhibitory immune checkpoint molecule to be clinically targeted to enhance T cell function. Like costimulatory CD28, CTLA-4 is also expressed on T cells; but unlike CD28, which is constitutively expressed on T cells, CTLA-4 expression is up regulated only after T cell activation and regulates early stages of T cell activation. Both CD28 and CTLA-4 bind to CD80 / CD86 on APCs but compared to CD28, CTLA-4 binds with much higher affinity. Therefore, expression of CTLA-4 on activated T cells induces a competitive inhibition of stimulatory CD28-CD80 / 86 signaling and inhibits T cell activation [15,16]. Functional studies on T cell activation suggest that crosslinking of CTLA-4 on TCR and CD28 stimulated T cells resulted in an anergic phenotype similar to that obtained when T cells are TCR stimulated in the absence of costimulatory signal. Specific pathways by which CTLA-4 suppresses T cell activation are still under investigation and it is suggested that activation of phosphatases downstream of CTLA-4 engagement with its ligands inhibits T cell activation. Critical role of CTLA-4 in T cell activation is best evident in ctla-4$^{-/-}$ mice, which exhibit a fatal lymphoproliferative and immune hyperactivation phenotype [17,18]. This provided convincing confirmation for blocking CTLA-4 expression and restoring function of activated T cells. Subsequently, various studies in human and animal models suggested that blocking CTLA-4 inhibitory signaling or "taking the brakes off" the immune cells restored T cell homeostasis.

Due to lethal effects in ctla-4$^{-/-}$ mice and absence of tumor specific CTLA-4 expression, CTLA-4 blockade did not originally appear to be a promising therapeutic strategy for cancer. However, Allisson et al demonstrated that partial blockade with CTLA-4 blocking antibody was beneficial in elimination of tumor growth with low toxicity in mice [19]. In poorly immunogenic tumors, combination of CTLA-4 blockade with GM-CSF based tumor vaccine showed better results as compared to CTLA-4 monotherapy alone [20]. In general, combination of CTLA-4 blockade with any methods that enhanced tumor antigen presentation (DNA or peptide based vaccines) yielded better results in many preclinical studies [21,22]. These preclinical observations led to the development of anti-CTLA-4 antibodies for clinical use.

Two fully humanized CTLA-4 blocking antibodies: Ipilimumab (MDX-010) and Tremelimumab (CP-675,206) are presently under clinical investigation. Ipilimumab was approved in 2011 at a dose of 3 mg / kg for treatment of un-resectable or metastatic melanoma by regulatory agencies in the United States and Europe [23]. Tremelimumab has been granted orphan drug status by FDA for treatment of malignant

Figure 2: CTLA-4 and PD-1 in regulation of anti-tumor T cell responses: (Upper Panel) Tumor antigen in the lymph nodes are presented by antigen presenting cells (APCs) via MHC to TCR which primes the T cell. APCs express ligands B7 which bind to the CD28 on T cells and enhances T cell activation. Activated T cells express CTLA-4 which also binds to CD28 but with much higher affinity. The tumor-specific T cell activation leads to proliferation and generation of T effector cells (Teff) which also express PD-1. (Lower Panel) PD-1+ Teff cells after trafficking to tumor microenvironment (TEM) encounter PD-1 ligands expressing tumor cells, which inhibit killing function of Teff cells. Thus, CTLA – 4 and PD-1 pathways are complementary to regulate antitumor Teff cells. Other suppressive cell types induced from TEM such as tumor associated macrophages (TAM), myeloid derived suppressor cells (MDSC), T regulatory cells (Tregs), further contribute to inhibit T cell response at site of tumor formation.

mesothelioma. Both antibodies have produced a good therapeutic response accompanied by immune related adverse events in treated patients [24]. Besides humanized monoclonal antibodies, CTLA-4 Ig fusion proteins (Abatacept, Belatacept) have also shown potent immunosuppressive properties in animal models of transplantation and autoimmunity [25-27]. CTLA-4 Ig is an approved therapy for rheumatoid arthritis [28,29] and clinical trials are currently in progress to assess its efficacy in transplantation tolerance, psoriasis and Crohn's disease.

Though CTLA-4 blockade proved to be beneficial only in a subset of cancer patients, yet it represented a giant leap for tumor immunotherapy. The adverse effects associated with CTLA - 4 blockade were as expected because blocking immune regulatory molecules can predispose the host to autoimmunity and hyper active immune responses. Interestingly, an important adverse immune event observed in patients of melanoma treated with CTLA-4 antibodies is development of antibodies against gut bacteria [30,31]. The correlates of immune protection or predictors of response after CTLA-4 blockade need to be clearly defined so that individual patients could be selected for CTLA-4 blockade.

PD-1 Pathway Blockade

The success of anti – CTLA-4 revolutionized the concept of targeting immune checkpoints to enhance anti -tumor activity. Another important inhibitory immune checkpoint molecule involved in regulation of T cell responses is PD-1 / PDL-1.PD-L2 pathway [32]. PD-1 belongs to CD28 family of immunoreceptors and is expressed on activated B, T and myeloid cells and tumor infiltrating lymphocytes. The two PD-1 ligands have differential expression; PD-L1 (also called B7H-1) is expressed on T

cells, B cells, macrophages and DC and is up regulated following activation of these cells [33,34]. In contrast, PD-L2 (also called B7-DC) expression is inducible on DC and macrophages [35].

Though the exact function of these ligands still needs to be elucidated, available data suggests that ligation of PD -1 to PD-L1 or PD-L2 triggers an inhibitory signaling pathway in the PD-1 expressing cells inhibiting T cell proliferation, cytokine production, and cell adhesion [33,36]. Similar to other CD28 family members, PD-1 transduces an inhibitory signal only when engaged in combination with T cell receptor (TCR) ligation, but not when cross-linked on its own. Both CTLA-4 and PD-1 have inhibitory effects on T cell activation however the timing of inhibition and signaling pathways differ for both the molecules. It is suggested that CTLA-4 inhibits immune responses in lymph node (during T cell priming phase) while PD-1 acts late at tissue sites (during the T cell effector phase) to limit T cell activation and avoid collateral damage [37]. Crucial role for PD-1 signaling has been best described in many models of chronic viral infection where exhausted T cells expressed high levels of PD-1 accounting for T cell dysfunction in chronic infection [38,39]. Similar to chronic infections, a comparable scenario of chronic antigen exposure in tumor microenvironment induces PD-1 / PD-L1 / PD-L2 expression in tumor cells leading to T cell exhaustion [40-43]. PD-1 expression was reported on tumor infiltrating lymphocytes and ligands for PD-1 were expressed on tumor cells of epithelial, non- epithelial and haematopoetic origin (Figure 2) [44]. Therefore, PD-1 signaling is an important pathway that induces impairment of T cell response in tumors and blocking this pathway could potentially liberate the T cells to perform effector functions [45].

A number of therapeutic antibodies that disrupt the PD-1 axis have entered clinical development. Although the various antibodies differ in structure, they can be broadly classified into two categories i) those that target PD -1(Nivolumab, Pidilumumab, MK3475 [46-48], ii) those that target PD-L1 (MPDL3280A; MEDI4736,; BMS-936559, MSB0010718C) [49-51],AMP-224 (Amplimmune). AMP-224 is a PD-L2 Fc fusion protein that is hypothesized to induce depletion of PD-1 positive T-cells representing exhausted effector cells.

Initial results with PD-1 blockade indicate a lower toxicity profile as compared to CTLA-4 blockade [52]. Certain immune related adverse events have also been described for patients treated with PD-1 and PD-L1 antibodies [52,53]. Overall, single agents have shown a modest response in tumor regression or improving overall survival. Since the nexus between tumor cells and immune system operates at multiple levels, combinatorial immunotherapy may be essential to break evasion mechanisms at multiple checkpoints. Combined immunotherapy with both CTLA-4 and PD-1 blockade in patients with melanoma has shown an accepted safety profile and better clinical activity as compared to monotherapy [54]. Recent data has also suggested that blocking CTLA-4 and PD-1 enhances anti-tumor response by ablating T regulatory cells [55,56]

Beyond CTLA-4 and PD-1 Pathway

Deciphering the basic mechanisms of T cell regulation in tolerance, inflammation and chronic infections has contributed to better understanding of other immune-checkpoints that are increasingly being characterized as targets for releasing the T cell brakes in cancer. As a result, the spectrum of immune-checkpoint targets is expanding beyond inhibitory receptors discussed above; numerous inhibitory ligands belonging to B7-family but with unknown receptors (B7-H3 and B7-H4) have been identified on tumor or tumor infiltrating cells and blockade of these in mouse models enhances anti-tumor immunity

Antibody	Molecule	Development Stage		
Nivolumab	Fully Hu-IgG4	US approved: Advanced Melanoma,		Squamous NSCLC after CT
		Ph* III (RCC, HNSCC, GBM, Gastric)		
Pembrolizumab	Humanized IgG4	US approved: Advanced Melanoma,		Squamous NSCLC after CT
		Ph III (RCC, HNSCC, GBM, Gastric)		
Pidilizumab	Humanized IgG4	Ph II multiple tumors (Pancreatic, CRC, RCC, Prostate, CNS)		
AMP-224	Fc – PD-L2-Fusion	Ph I		
MPDL3280A	Engineered Hu IgG1	Ph III		
MSB0010718C	Fully Hu IgG1	Ph III		

Table 2A: Clinical Development of Anti-PD-1 Checkpoint Inhibitors.

Target	Antibody	Molecule	Development
CTLA-4	Ipilimumab	Humanized IgG1	Approved: Advanced Melanoma, Ph III (RCC, NSCC, GBM, SCLC)
	Tremelimumab	Fully Hu IgG2	Ph III (NSCLC, HNSCC)
	INCB024360	Small Molecule Inhibitor	Ph I
IDO	NLG919	Small Molecule Inhibitor	Ph I
	MDX-1105	Fully Hu IgG4	Ph I
PD-L1	MEDI4736	Engineered Hu IgG1	Ph III
	MPDL3280A	Humanized IgG	Ph II; Ph III
LAG-3	BMS-986016	-	Ph I
	IMP321	LAG3-Ig	Fusion protein
Ph II (Breast cancer)			
B7-H3	MGA271	Humanized IgG1	Ph I
B7- H4	-	-	Preclinical
TIM-3	-	-	Preclinical

NSCLC - Non small cell lung cancer; RCC - Renal cell carcinoma; HNSCC - Hean and neck squamous cell carcinoma; GBM - Glioblastoma; CRC - Colon rectum cancer; SCLC - Small cell lung cancer

Table 2B: Clinical Development of Other Checkpoint Inhibitors Target Antibody.

[57]. Another inhibitory checkpoint molecule in the same category as CTLA-4 and PD-1 is LAG-3, which inhibits T cell proliferation, function [58-61] and contributes to the suppressive action of T regulatory cells (Tregs) [62]. Dual blockade of both PD-1 and LAG-3 has been shown to restore tumor specific immune response and enhance survival in murine models of tumor [58]. Currently clinical trials are underway to determine the safety and efficacy of combinatorial therapy with anti-LAG-3 antibody with or without PD-1 blockade in solid tumors (trial ID CA224-020, NLM Identifier NCT01968109). Apart from immune checkpoints, metabolic checkpoints such as inhibitor compounds for enzymes like indoleamine 2,3-dioxygenase (IDO), isocitrate dehydrogenase, adenosine signaling etc are also an emerging target for development of anti-cancer therapeutic molecules [63-65]. Tumor microenvironment presents many metabolic challenges which may contribute to a rewiring of anti-tumor T cell response. This new area of immunometabolism will certainly add new dimensions to manipulate T cell function; we are already noticing an exponential information explosion in this arena as well. This may open up entirely new avenues to treat immune mediated disorders. Combination of immune checkpoints which boost the immune response and metabolic checkpoints which provide a host friendly tumor microenvironment may also be one combinatorial approach in cancer therapy.

The targets for which biological or small molecule inhibitors are currently available are detailed in the Table 2, but the list is not comprehensive. Tumor immunotherapy has seen a dramatic transition from the era of Coley's toxin [66] to immune checkpoints. Nevertheless, substantial data show that immunotherapy does not follow "one size fits all" approach and predictors of response to therapy need to be identified so that clinicians can selects patients for particular monotherapy or combination immunotherapy. Blocking a single molecule has not produced a completely curative response thus underscoring the importance of multiple, probably, redundant molecules working in tandem to promote immune escape of tumor cells. While it is possible that there are many other molecules still to be discovered there is substantial evidence to suggest that combinatorial therapy involving immune, molecular and metabolic checkpoints and not monotherapy alone might be the ideal way to develop completely curative and specific immune-therapeutic modalities.

Conclusion

Exploiting the immune system against tumor cells has been considered an attractive therapeutic option, successive failures or limitation of practical usage of various immune therapeutic approaches resulted in the loss of creditability of cancer immunotherapy. With the better understanding of T cell activation and regulation and its successful translation towards development of broad spectrum anti-cancer agents in form of immune checkpoint inhibitors has revived the immune therapy field. However, this novel treatment which engages patient's immune response to target tumor cells needs to be integrated with conventional approaches as surgery, chemotherapy, radiation therapy and targeted therapy which directly attack cancer cells. Furthermore, achieving maximum clinical benefit from immunotherapeutic molecules may also require a careful investigation of extent of cooperatively between different immune checkpoints. It might also be important to contemplate combination treatments that can augment both innate (NK cells, γδ T cells etc) and adaptive arm of host immune system in tumor microenvironment for better clinical benefit.

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