

# Costimulation, Coinhibition and Cancer

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**Abstract:** The immune system is an important defense mechanism against cancer and is often dysfunctional in patients with malignancies. The central regulator of the anti-cancer adaptive immune response is the T lymphocyte. T lymphocyte activation requires the completion of a carefully orchestrated series of specific steps that can be preempted or disrupted by any number of critical events. Particularly important is the provision of a costimulatory signal, the binding of accessory molecules on the antigen presenting cell to receptors on the T lymphocyte. Though costimulatory signals were traditionally envisioned as T lymphocyte-activating events, recent discoveries have highlighted their duality: they can be either stimulatory (costimulation) or inhibitory (coinhibition). In this article we review costimulation and coinhibition as potential targets for cancer therapy. We begin by presenting a general framework for thinking about the immune system in the context of cancer. Our discussion then bridges the various aspects of immune dysfunction seen in cancer with the presence of coinhibitory (ex: PD-1, PD-L1, CTLA-4, BTLA) and costimulatory (ex: CD28, ICOS, 4-1BB, CD40, OX40, CD27) signaling. Lastly, we develop a model of cancer-related immune dysfunction that parallels the concept of immunoediting. Throughout the article we emphasize clinically relevant research often applicable—but not limited—to the example of renal cell carcinoma.

**Keywords:** Costimulation, coinhibition, tumor immunology, immunoediting, immunotherapy, T lymphocyte.

## INTRODUCTION

The history of cancer therapy is filled with failed treatments and unpreventable patient death. Fortunately, new discoveries are bringing new possibilities for patients with cancer, particularly in the form of immunotherapy. The future of cancer treatment is bright, and we believe that immunotherapy will see much more of its potential demonstrated in the next decade.

In this review we discuss one of the newest discoveries in tumor immunology: coinhibition. This chapter will first introduce key concepts of T cell function and tumor immunology. We then describe some of the key defects in immunity that are present in cancer. Lastly, we propose a model to explain why certain tumors are eliminated by the immune system and others are not. Though we will discuss many different tumor types in this article, the readers will notice that we often return to the example of renal cell carcinoma (a.k.a. kidney cancer). There are three good reasons for this. First, renal cell carcinoma is a tumor that has been treated with multiple immunotherapeutic modalities, both with success and failure. Second, there is an abundance of research describing the role and function of the immune system in this tumor. Third, a major proportion of our collective research experience has focused on the immunology of renal cell carcinoma.

## TUMOR IMMUNOLOGY: A BRIEF INTRODUCTION

Human cells are continuously dividing, differentiating and dying: acts that are orchestrated by a highly redundant bureaucracy of cellular control mechanisms that are in place

to ensure the stability of growth in bodily tissues. Like all large bureaucracies, the body's safeguards occasionally fail, allowing individual cells to escape the control mechanisms that normally govern their existence and proliferate or specialize in an inappropriate manner.

## Immune Surveillance and Immunoediting

It has been nearly 100 years since Paul Ehrlich first suggested that the immune system plays an active role in finding and eliminating newly developing cancer cells [1]. This concept was not widely accepted until nearly 50 years later when Burnet and Thomas revived it and coined the term "*immune surveillance*" [2,3]. The implication of immune surveillance is that the immune system is on constant alert and actively seeks out and destroys malignant cells as they are formed. Although immune surveillance was an attractive proposal in the 1950s and 1960s, establishing its existence *in vivo* proved to be a difficult matter, and a series of experiments conducted in the 1970s provided early evidence that immune surveillance might not actually exist [4,5]. A key initial observation made by several groups was that cancer did not arise more frequently in athymic nude mice that lacked an intact immune system than in immunocompetent mice—a finding that ultimately led to the early abandonment of the immune surveillance hypothesis in the late 1970s. Auspiciously, a few stalwart proponents of the theory remained and an influx of new knowledge regarding natural killer cells [6],  $\gamma\delta$ -T lymphocytes [7], perforin [8], interferons [9], and other components of the immune system, has rekindled interest in immune surveillance.

The theory of immune surveillance is currently experiencing a rebirth and is emerging in a more complex form than it originally had [10]. The term "*immunoediting*" has been proposed to stress the duality of immune

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surveillance—it is both a host-protecting and a tumor-sculpting process [11]. Dunn, Old and Schreiber have published a series of enlightening articles in which they put forward a 3-part model of cancer immunoeediting that encompasses tumor elimination, host-tumor equilibrium and tumor escape [11-13]. They argue that cancer immunoeediting is a broad process that involves both adaptive and innate immunity and could lead to either tumor rejection or tumor tolerance. This model is very similar to ideas presented by Stutman over 20 years ago [14]. Much of what we will discuss in this manuscript fits nicely alongside this paradigm.

### Immune Tolerance: Central and Peripheral

The immune system has two broad arms: the *innate immune system* that responds non-specifically to threats and the *adaptive immune system* that is responsible for precise, antigen-specific, targeted immune attacks. The principal effector cells of the adaptive immune system are lymphocytes that have antigen-specific receptors on their cell surface. These receptors are randomly generated by early somatic rearrangements in the lymphocyte receptor genes, a process that occasionally results in the formation of a self-reactive receptor that could theoretically target self antigens and result in autoimmune disease [15,16]. *Central tolerance* is the ability to identify and destroy those lymphocytes with self reactive receptors early in their development, before they can cause any trouble. The end result of central tolerance is the formation of a population of lymphocytes (B and T cells) with a plethora of receptors designed to detect non-self antigens and be tolerant to self antigens.

Central tolerance is not perfect, however, and lymphocytes with problematic antigen receptors occasionally slip into the bloodstream, with the potential to cause autoimmune disease. Fortunately, a second process of tolerization has evolved to address this problem. *Peripheral tolerance*, tolerance occurring at any site where a lymphocyte encounters antigen, can arise due to a number of circumstances [17]. In fact, in order for a T cell to respond to a particular antigen, it must pass three critical tests (similar to marriage). First, several TCRs on a particular T cell must bind avidly to antigen-MHC complexes on an antigen presenting cell (APC), thereby demonstrating the desire for a relationship [18]. Second, costimulatory molecules on the APC must bind to their receptors on the T cell, confirming that the offer has been accepted [19]. Lastly, a permissive biochemical environment must be present for the courtship to proceed expeditiously [20]. A problem at any one of these steps can produce a tolerizing signal that leads to one of two basic fates: death of the T cell by apoptosis or the induction of the unresponsive state of T cell anergy [21-23]. These events can result in the inactivation or death of an entire family of T cell clones that express a particular TCR.

Other mechanisms of peripheral tolerance also exist. *Immunologic ignorance*, refers to the situation where the T cell is never activated simply because it never encounters enough antigen on the surface of an APC to force the activation process to begin [24]. Immunologic ignorance appears to be an antigen presentation problem and not the result of apoptosis or anergy. At the opposite end of the spectrum is *clonal exhaustion* (a.k.a. replicative senescence),

a process where T cells that are continuously exposed to abundant antigen replicate incessantly to the point of burnout [25]. Some groups have been able to rescue these T cells from burnout by targeting the coinhibitory pathways discussed below [26]. Lastly, a specialized subpopulation of T cells—known as *regulatory T cells*—has the ability to cause antigen-specific immunosuppression and tolerance [27].

There are several types of regulatory T cells ( $T_{reg}$  cells) and we would like to familiarize readers new to this subject to a few important points [27,28]. T cell subsets were first shown to suppress immune responses in the 1970s, and they were called *suppressor T cells* [29]. The failure to identify antigen-specific factors and unique cell surface molecules that caused immune suppression led to stagnation of this field. Interest was rekindled in the mid-1990s when Sakaguchi *et al.* demonstrated that a small population of CD4<sup>+</sup> T cells that co-expressed the IL-2 receptor  $\alpha$ -chain (CD25) could control autoreactive CD4<sup>+</sup> T cells *in vivo* [30]. *In vitro* murine studies have since confirmed that certain CD4<sup>+</sup>CD25<sup>+</sup> T cells can function as potent inhibitors of T-cell mediated immunity [31], and subsequent studies have found similar T cell populations in humans [32].  $T_{reg}$  cells constitute a family of specialized T cells that can be divided into two broad subsets: natural and adaptive. The *natural  $T_{reg}$*  cell population develops as a separate T cell lineage in the thymus [33]. These cells are antigen-specific and populate the periphery to guard against autoimmune reactions [33]. The second subset of *adaptive  $T_{reg}$*  cells appears to develop from mature, peripheral CD4<sup>+</sup> T cells in response to tissue-specific or foreign antigens, and mediate regulation through the release of soluble cytokines (IL-10). A major problem with the study of  $T_{reg}$  cells has been the lack of specific cell markers that distinguish them from other lymphocytes. Recently, the ability to characterize  $T_{reg}$  cells has improved with the identification of the transcription factor Foxp3 and the surface marker CD127 [34,35]. Murine and human natural CD4<sup>+</sup>CD25<sup>+</sup>  $T_{reg}$  cells express high levels of FoxP3 and low levels of CD127.

Although research into the impact of regulatory T cells on human cancer has been limited, several key studies have emerged in recent years demonstrating their importance. Cancer patients appear to have increased numbers of  $T_{reg}$  cells [36]. Murine studies have shown that antibody-mediated depletion of CD25<sup>+</sup> cells can enhance tumor immunity and rejection [37]. Curiel *et al.* were the first to demonstrate a direct link between  $T_{reg}$  cells and human tumor immunopathogenesis by showing that tumor-infiltrating  $T_{reg}$  cells block tumor-antigen specific effector T cell responses and correlate with poorer prognosis [38]. Work in our laboratory suggests that  $T_{reg}$  cells also play an important role in RCC (unpublished results).

### T-Cell Activation

As part of the maturation/selection process, a T cell is committed to one of two lineages: the CD4<sup>+</sup> T helper cell lineage ( $T_H$ ) that is MHC class II (MHC<sub>II</sub>) restricted or the CD8<sup>+</sup> cytotoxic T cell ( $T_C$ ) lineage that is MHC class I (MHC<sub>I</sub>) restricted. The antigen receptors of both lineages are similar in that they can bind to any MHC molecule (class I or class II) that presents an appropriate antigen. It is in fact

the coreceptors, CD4 and CD8, that determine the MHC specificity of the T cell because they bind exclusively to MHC<sub>II</sub> and MHC<sub>I</sub>, respectively.

Once they have matured in the thymus, T<sub>H</sub> and T<sub>C</sub> cells are released into the bloodstream in search of antigen. These newly minted T cells are naïve in the sense that they have never encountered the antigen that their TCRs were built to recognize. The fate of the naïve T cell is set by two determinants: the type of coreceptor it expresses and the nature of its first contact with its antigen. The relationship between the T cell and the antigen-bearing APC is critical to understanding how cancers can trick the immune system into a state of unresponsiveness.

### Signal 1: Antigen Presentation to the T Cells

For naïve T<sub>H</sub> cells, activation requires the presence of a mature dendritic cell (DC) with antigen-loaded MHC<sub>II</sub>. Contrarily, the activation of a T<sub>C</sub> cell requires a target cell, an APC with antigen-loaded MHC<sub>I</sub>, and an antigen-specific effector T<sub>H</sub> cell for cytokine support. Activation of a T cell therefore requires an orchestrated team effort which is not a chance occurrence.

For the T cell to be activated by an MHC-bound target antigen, several things must occur. First, *cell adhesion molecules* must be present on both the APC and the T cell. The cell adhesion molecules serve two main purposes: to home the T cell to the APC and help these two cells remain in contact long enough to allow as many TCRs on the T cell as possible to become activated. It can take as long as 30 hours and involve the binding of roughly ~100 MHC-antigen complexes to ~20 000 TCRs to activate a T cell [39]. Second, the formation of an *immunologic synapse* must occur. The formation of the immunologic synapse can be visualized with microscopy as a 3-dimensional triple-ringed structure called the *supramolecular activation complex* (SMAC) [40]. The SMAC is formed due to changes in the actin cytoskeletons of the T cell and APC and has been shown to contain TCRs, MHC-antigen complexes, the CD4 and CD8 coreceptors, and enzyme-bearing plasma membrane lipid rafts, all of which are required for T cell activation [41]. Lastly, after binding the right MHC-antigen combination, the TCR is phosphorylated and an appropriate signal is directed into the T cell for processing. This signal results in both TCR internalization and downregulation. The remaining TCRs in the SMAC compete with the internalization process to determine the fate of the T cell. If enough TCRs bind to MHC-antigen complexes, the T cell lives to test itself at signal II. If the internalization process dominates, the T cell will not proceed to complete activation. This control mechanism ensures that an over-exuberant T cell response does not result every time a T cell encounters its antigen.

### Signal 2: Costimulation and Coinhibition

After receipt of the first signal, the T cell must receive a second confirmatory signal if it is to avoid anergy or apoptosis. The molecules that give this second signal are called *costimulatory molecules*, and are numerous and varied in their characteristics (see Table 1) [42]. Certain molecules (ex: CD28) give the T cell the activation/proliferation signal

(*costimulation*) it desires, while others (ex: CTLA-4) do exactly the opposite (*coinhibition*). Some costimulatory molecules can be either stimulatory or inhibitory [43].

The principal costimulatory molecule is CD28, a receptor that is constitutively expressed on the surface of nearly all CD4<sup>+</sup> T<sub>H</sub> cells and the majority of CD8<sup>+</sup> T<sub>C</sub> cells [19,42]. When both the TCR and CD28 bind to their ligands at the same time, their respective intracellular signals act synergistically to activate the cell's replicative machinery and secretory apparatus (see Fig. 1A). Successful delivery of signal 2 implicates migration of a coinhibitory receptor called CTLA-4 from its subcellular Golgi compartment to the T cell plasma membrane. CTLA-4 is normally not present to any significant degree in the inactive T cell membrane, competes ravenously with CD28 for its ligands (B7.1 and B7.2), and sends a turn off signal to the T cell. Therefore, the newly activated T cell has to manage two contradictory communications: an early activation/proliferation message from CD28 and a delayed deactivation/nonproliferation message from CTLA-4. Recent evidence suggests that when B7-1 and B7-2 bind to their receptors, signals are generated in both directions: into the APC and into the T cell, affecting both cells [44,45].

More recent years have brought the discovery of several new costimulatory molecules. Some of these costimulatory molecules are part of the B7/CD28 family (ex: B7-H1, B7-DC, ICOSL, B7-H3, B7-H4) while others are members of the tumor necrosis factor receptor (TNF/TNFR) family (ex: CD27, CD40L, OX40, HVEM and 4-1BB) [42,46]. In general, members of the TNFR family act in a similar fashion to CD28, stimulating T cell proliferation and cytokine secretion. Contrarily, the physiologic actions of the newest B7 family members are varied.

ICOS (a.k.a. inducible costimulator) is a receptor whose expression is induced in the T cell following binding of either the TCR to MHC-antigen complex or CD28 to B7 [47,48]. It binds to ICOSL (a.k.a. B7h, GL50, B7RP-1, LICOS, ICOS-L, B7-H2, KIAA0653, CD275) that is constitutively expressed in lymphoid tissue [49-51]. Although the ICOS/ICOSL pathway provides positive costimulation, it is distinct from the CD28/B7 pathway [49]. For instance, it does not appear to be involved in immediate T cell IL-2 release but is involved in the differentiation of T<sub>H</sub> cell subtypes (discussed later) and in providing help to B cells [47,52,53].

PD-1 (a.k.a. programmed death 1) is a receptor found on T cells and numerous other cell types, including: thymocytes, B cells, monocytes, endothelial cells and NK-T cells [54]. An overwhelming amount of evidence suggests that PD-1 signals inhibit T cell activation and proliferation, although some studies have suggested that it can be stimulatory as well (see Fig. 1B) [55-57]. PD-1 has two known ligands, PD-L1 (a.k.a. B7-H1) and PD-L2 (a.k.a. B7-DC) [55,56]. PD-L2 appears to have a higher affinity for the PD-1 receptor than PD-L1 and its expression is induced on dendritic cells and macrophages by the presence of IL-4 [58,59]. Contrarily, PD-L1 is expressed on T cells, B cells and macrophages in response to inflammatory cytokines such as IFN $\gamma$  [59]. T<sub>H</sub>1 responses favor PD-L1 expression whereas T<sub>H</sub>2 responses favor PD-L2 expression. PD-L1 also differs from PD-L2 in that it is found in many tissues

**Table 1. Major Costimulatory Molecules and their Actions**

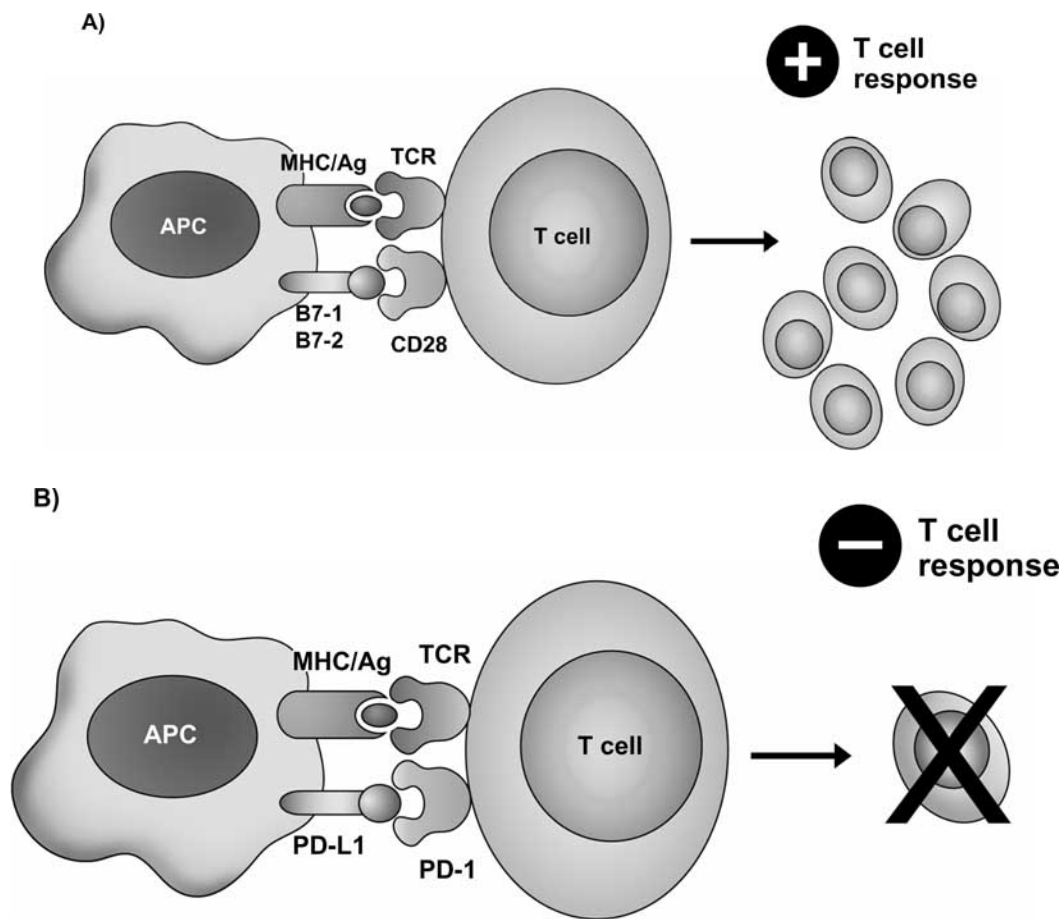
	Receptors (T cell)	Ligands (APC)	Major Cellular Effects
B7/CD28 Family	CD28	B7-1 (CD80)	-Constitutively expressed on most T cells -T cell activation and proliferation ( $\uparrow$ IL-2 and IL-2R) -Differentiation ( $\uparrow$ IL-1, IL-4, IL-5, IL-6, IL-10, TNF, IFN $\gamma$ )
		B7-2 (CD86)	-Block apoptosis ( $\uparrow$ Bcl-xL) - $\downarrow$ TCR signalling threshold
	CTLA-4(CD152)	B7-1 (CD80)	-Induced 48-72 hours after TCR signalling (signal I) -Competes with CD28 for its ligands
		B7-2 (CD86)	-Blocks T cell activation and proliferation ( $\downarrow$ IL-2) -Cell cycle arrest
	PD-1	PD-L1 (B7-H1)	-Blocks T cell activation and proliferation ( $\downarrow$ IL-2)
		PD-L2 (B7-DC)	-Cell cycle arrest
	ICOS	ICOSL (B7-H2)	-Inducible molecule found only on activated T cells -B cell development and germinal center formation ( $\uparrow$ IL-4, IL-10, CD40L) -T <sub>H</sub> 2 differentiation of T <sub>H</sub> cells ( $\uparrow$ IL-4, IL-10)
	?	B7-H3	-Blocks T cell activation and proliferation (T <sub>H</sub> 1 > T <sub>H</sub> 2)
	?	B7-H4	-Blocks T cell activation and proliferation ( $\downarrow$ IL-2, IL4, IL-10, IFN $\gamma$ ) -Cell cycle arrest
BTLA	HVEM	-Blocks T cell activation and proliferation	
TNF/TNFR Family	CD40L	CD40	-T cell activation and proliferation
	4-1BB (CD137)	4-1BBL	-T <sub>C</sub> cell activation and proliferation -T <sub>C</sub> > T <sub>H</sub>
	OX40	OX40L	-T cell activation and proliferation -T <sub>H</sub> > T <sub>C</sub>
	CD27	CD70	-T cell activation and proliferation -2° > 1° immune responses (favors immune memory)
	HVEM	LIGHT	-T cell activation and proliferation

unrelated to the immune system [60], it induces apoptosis [61], and it appears to be a major player in inflammation and autoimmunity [62,63]. The differences in physiologic action observed between PD-L1 and PD-L2 can be rationalized in four ways: (1) the two molecules probably bind to different sites on PD-1 [64]; (2) there are probably receptors other than PD-1 for these molecules [65]; (3) bidirectional signaling may cause changes that are ligand-specific, and (4) PD-1's immunoreceptor tyrosine-based switch motif (ITSM) may interact with different phosphatases within the T cell, which implies that the way that the PD-1 signal is received inside the T cell may vary based on the state of activation of the T cell [66].

*BTLA* (a.k.a. B and T lymphocyte attenuator) is a receptor found on activated B cells and T cells that has many structural and functional similarities to PD-1 [67]. It was originally assumed that *BTLA* would bind to B7-H4. However, recent evidence has shown that *BTLA*'s ligand is

not a member of the CD28/B7 family but a member of the TNF/TNFR family called *HVEM* (a.k.a. herpes virus entry mediator) [68,69]. This discovery demonstrates crosstalk between structurally dissimilar families of costimulatory molecules and that costimulatory and coinhibitory signals can be competitive, and perhaps even coordinated, through a single receptor.

*B7-H3* (a.k.a. B7RP-2) is a ligand found on APCs and other cell types that binds to an unknown receptor on the T cell [70]. Studies evaluating the precise physiologic role of *B7-H3* are conflicting: some groups found evidence suggesting that *B7-H3* has positive costimulatory properties [70], other groups have found the exact opposite [71,72]. There may be functionally distinct *B7-H3* splice variants or antagonistic *B7-H3* receptors [73]. Inflammatory cytokines such as IFN $\gamma$  induce *B7-H3* expression on macrophages and monocytes [70].



**Fig. (1).** **A)** Costimulation induces T cell activation. **B)** Coinhibition induces T cell anergy or apoptosis.

*B7-H4* (a.k.a. *B7S1*, *B7x*) is found on a wide variety of tissues (including APCs) and binds to an unidentified receptor [74]. No controversy exists as to the function of *B7-H4*: it inhibits the activation and proliferation of T cells [74-76]. *B7-H4* appears to bind a receptor that is present on activated, but not naive T cells [75,76].

In summary, there are two large families of costimulatory molecules: the TNF/TNFR family and the B7/CD28 family (see Fig. 2). TNF/TNFR costimulatory molecules tend to activate the T cell and induce its proliferation. The B7/CD28 family is heterogenous: CD28 and ICOS are predominantly positive costimulators while CTLA-4, PD-1, BTLA and the unidentified *B7-H4* receptor are predominantly coinhibitory molecules.

**Signal 3: Cytokine Release**

After receiving signals I and II, the T cell is ready to transcribe dozens of genes whose protein products are required for division and differentiation. Some of these genes are activated *immediately* (within minutes) whereas others are activated either *early* (hours) or *delayed* (days). Key cytokines that are implicated are IL-2, IL-4, IL-7, IL-12 and IL-15 [77,78].

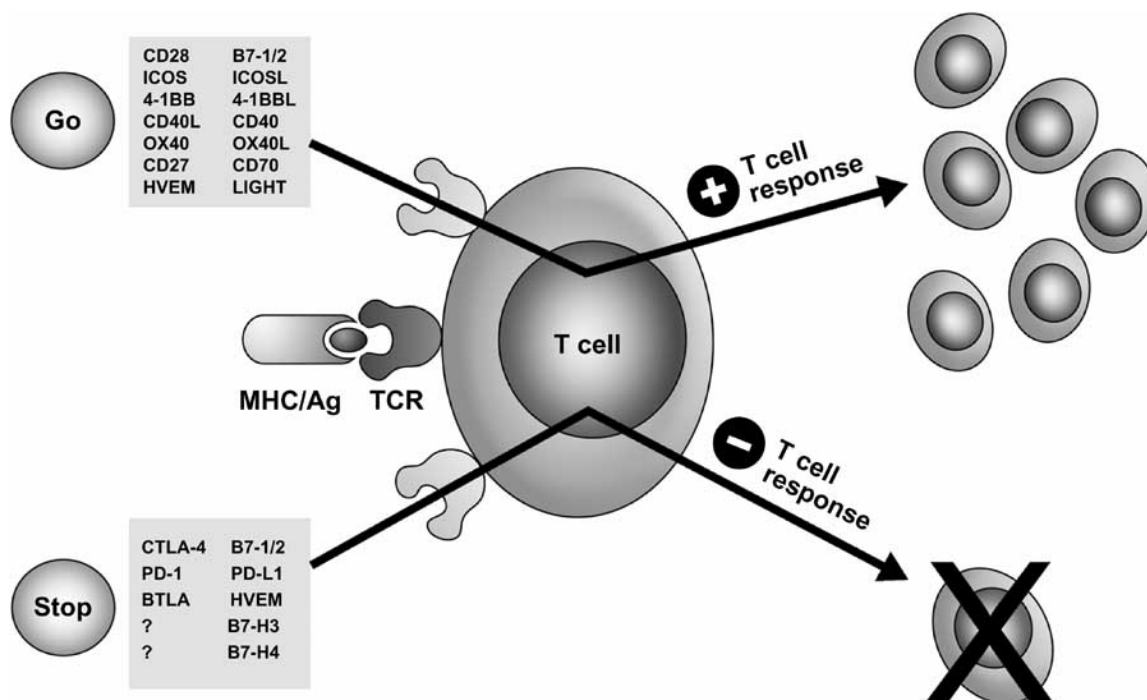
Perhaps the most important cytokine that is transcribed immediately following signals I and II is *interleukin 2* [79]. IL-2 can act in autocrine (stimulate itself) or paracrine

(stimulate others nearby) fashions to initiate T cell proliferation. Although IL-2 stimulates the proliferation of all T cell subsets, it tends to have its most pronounced effect on  $T_H$  cells. *T helper cells* are particularly important for CD8+  $T_C$  cells because *T cytotoxic cells* tend to have difficulty generating a strong enough IL-2 signal on their own to induce proliferation. IL-2 is also crucial for the development of self-tolerance [80]. The  $\alpha$ -chain of the IL-2 receptor is CD25 and it is normally expressed only on activated T cells. The constitutive expression of CD25 on an unactivated T cell is indicative of an inhibitory regulatory T cell phenotype [30].

*Interleukin 4* (IL-4) is involved in the differentiation of  $T_H$  cells into specific subsets, in particular the  $T_H2$  subset [81].  $T_H2$  cells are important for fighting extracellular pathogens and ensuring that B cells are producing the appropriate isotype of antibody [82].

*Interleukin 7* (IL-7) is a key regulator of early lymphocyte development in the bone marrow and thymus [83]. It is involved in TCR gene rearrangement and favors the development of a different type of TCR, the  $\gamma\delta$ -TCR [84]. Memory T cells are also induced and maintained by IL-7.

*Interleukin 12* (IL-12) induces differentiation of  $T_H$  cells into  $T_H1$  subset [85], which are important for combating intracellular pathogens, antagonizes IL-4 and inhibits the differentiation of  $T_H2$  cells [82].



**Fig. (2).** A multitude of costimulatory and coinhibitory molecules can influence T cell function and the anti-cancer immune response.

Not only is *interleukin 15* (IL-15) critical for the formation of natural killer (NK) cells, but it is also very important for lymphocyte recirculation in the body and homing to the lymph nodes. IL-15 is involved in promoting the differentiation of effector T<sub>C</sub> cells (i.e. cytotoxic T cells, CTLs) and CD8<sup>+</sup> memory T cells [86].

### LEUKOCYTE DYSFUNCTION IN CANCER

There is an abundance of research that suggests that the immune system is often dysfunctional in patients with cancer [87]. Certain cancers, such as renal cell carcinoma, seem to have more prominent defects in immunity than other tumors. In this section we discuss the evidence that the immune system may be malfunctioning in the context of cancer.

#### Tumor-Infiltrating Leukocytes

If the immune system can target and kill tumor cells, it seems logical that tumors filled with leukocytes would be less likely to progress than tumors without tumor-infiltrating leukocytes. This is akin to escaping a room full of assassins trained to kill you. Many researchers have shown the presence of intratumoral leukocytes in histologic tumor sections to be a favorable prognostic feature in breast cancer [88,89], lung cancer [90,91], colorectal cancer [92,93], stomach cancer [94,95], esophageal cancer [96,97], gallbladder cancer [98,99], malignant melanoma [100,101], neuroblastoma [102,103], cancers of the head and neck [104], prostate cancer [105], testis cancer [106,107], bladder cancer [108,109], and kidney cancer [110]. Unfortunately, the story is also obfuscated by a number of manuscripts that have shown the opposite effect in breast cancer [111,112], malignant melanoma [113,114], lung cancer [115,116], bladder cancer [117], and renal cell carcinoma [118,119].

Why are these results so divergent? Part of the answer may be explained by the fact that not all tumor-infiltrating immune cells are the same in terms of composition and states of activation. A critical first step in understanding the immunobiology of a given tumor typically arises from analyses of what populations and subsets of immune cells are actually infiltrating the tumor. Specialized techniques such as immunohistochemistry, flow cytometry, cell culture and functional assays are often required because routine techniques cannot distinguish between many related cell types nor can reveal the functional state of an immune cell. Another reason why studies may diverge in their interpretation of the importance of tumor-infiltrating leukocytes is the frequent lack of thoroughness in controlling for other recognized prognostic factors. Studies that employ sound methodology, adjusting for confounding predictors (such as tumor grade and stage), are less likely to reveal artifactual prognostic associations with a novel predictive marker. In the specific case of RCC, we are aware of 13 published prognostic tools for predicting survival [120-132], all of which were rigorously designed, and none of which incorporated the histologic presence of tumor-infiltrating lymphocytes as part of their scoring system. One large and adequately controlled study demonstrated that mononuclear cell infiltration in histologic RCC sections is associated with poorer cancer-specific survival, even after controlling for all other important and well-described prognostic factors [119].

#### T Lymphocytes in Renal Cell Carcinoma

It has been clearly shown that the T cells infiltrating RCC tumors differ from T cells observed circulating within the peripheral bloods of corresponding patients. For instance, although the absolute quantity of tumor-infiltrating T cells observed in RCC specimens increases in parallel

with both tumor stage and grade [133], the relative proportion of activated intratumoral T cells actually diminishes [134]. This suggests that although aggressive RCCs recruit many T cells to the tumor site, these tumors are somehow capable of preventing the T cells from being activated. It has also been shown that T cells infiltrating RCC tumors frequently exhibit dysfunctional T cell receptors [135,136].

If we now only consider the specific CD8<sup>+</sup> T cell subset, it has been shown that the quantity of tumor-infiltrating CD8<sup>+</sup> T cells increases with severity of tumor grade and tumor proliferative activity [134,137]. Some groups have even shown that a high level of intra-tumoral CD8<sup>+</sup> T cells predicts an increased probability of RCC recurrence [133], a poor response to IFN $\alpha$  immunotherapy [110], and a shorter cancer-specific survival [137]. In contradistinction, one group noted that an increased quantity of intra-tumoral CD8<sup>+</sup> T cells predicted a more favorable outcome when infiltrating T cells were actively proliferating [137]. Taken together, these data suggest that the mere presence of CD8<sup>+</sup> T cells within an RCC tumor is insufficient to produce an antitumoral immune response. Rather, it appears that CD8<sup>+</sup> T cells need to be appropriately activated in order to effectively mediate tumor regression. These data also raise the specter of tumor-related factors that prevent CD8<sup>+</sup> T cells from being fully activated as well as the possible existence of regulatory/inhibitory CD8<sup>+</sup> T cell subsets [138].

In the case of the CD4<sup>+</sup> T cell subset, the results pertaining to RCC are much more heterogeneous. While some researchers have found increased CD4<sup>+</sup> T cells in high grade and high stage tumors [118,139], others have reported the opposite [140]. One group showed that the response to IFN- $\alpha$  immunotherapy tended to be better in patients with high intratumoral CD4<sup>+</sup> cell counts and, perhaps, thereby improving survival [110]. Contrarily, another group reported that RCC-specific survival was diminished in patients with high intra-tumoral CD4<sup>+</sup> T cell counts [118]. Clearly, not all CD4<sup>+</sup> T cells are the same. An interesting and relevant finding in other experimental and clinical tumors has been the observation that CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells may encompass one entity that inhibits CD8<sup>+</sup> effector T cell function [141,142].

### Natural Killer (NK) Cells in Renal Cell Carcinoma

Natural killer cells comprise an important fraction of the immune cells that infiltrate renal cell carcinomas and are typically of the CD16<sup>bright</sup> cytotoxic-effector variety [134,143]. The more NK cells that are found in a RCC, the more active these NK cells appear to be [143]. The number of tumor-infiltrating NK cells may decrease as the tumor grade increases, possibly indicating that aggressive tumors tend to attract less NK cells [134]. We are aware of no study that has shown NK cells to be of prognostic value in RCC, though such studies do exist for other cancers [144-146].

### Neutrophils, Monocytes and Macrophages in Renal Cell Carcinoma

An awareness has slowly developed that inflammation and the innate immune system play an important role in

cancer development [147]. In the case of RCC, markers of inflammation have been long known to predict a poor outcome [148]. A few clinical trials have also shown that high levels circulating or tumor-infiltrating inflammatory cells such as neutrophils and macrophages- predict a poor response to IL-2 immunotherapy [121,149]. Interestingly, one group showed that a drop in monocytes occurring during IL-2 therapy predicted a favorable treatment response [150]. One explanation of why inflammation might impair antitumoral immunity pertains to the interaction of such cells with T cells [151]. It has been speculated that part of the dysfunctional phenotype of intratumoral NK and T cells may result from oxidative stress or other forms of damage directly caused by products secreted by activate tumor-associated neutrophils and macrophages cells [152]. Thus, clinical trials combining IL-2 and histamine (an inhibitor of hydrogen peroxide formation and release) have been conducted to treat advanced RCC but, to date, these trials have not shown a clear benefit resulting from the addition of histamine to the treatment regimen [153].

## COSTIMULATION AND COINHIBITION IN CANCER

As previously discussed, T cells exhibit a variety of costimulatory and coinhibitory receptors and ligands that are capable of triggering distinct and occasionally competing functions (see Table 1). Though most of these molecules have not been systematically evaluated in cancer, the few that have been studied have revealed some remarkable results. In this section we will contrast the roles of costimulation and coinhibition in the specific context of cancer.

### Evidence from Renal Cell Carcinoma

It has been known for some time that positive costimulation has significant effects on RCC-associated immunity. For instance, transfecting RCC cells to constitutively express the costimulatory ligands, B7-1 and B7-2, results in significantly amplified antitumoral immune responses [154-156]. Hence, this approach has been exploited in a phase I clinical trial that tested genetically modified RCC cells expressing high levels of B7-1 as a tumor vaccine co-administered with IL-2 [157]. Also, high levels of B7-1 expression in patients with metastatic RCC predicts a favorable response to nephrectomy and IL-2 immunotherapy [158]. Similarly, IL-2 immunotherapy appears to cause RCC tumors to upregulate their expression of B7-1 and [159,160], in addition, a phase I clinical trial has also shown that CD28-activated T cells can serve as a form of adoptive T cell therapy for RCC patients [161]. There is much less known about the role of CTLA-4 in RCC. One study has reported that RCC patients harbor increased levels of CTLA-4 positive lymphocytes and that these lymphocytes are phenotypically and functionally consistent with regulatory T cells [36]. Interestingly, those patients that responded to IL-2 immunotherapy were also patients in which CTLA-4 positive T<sub>Reg</sub> cell levels declined during treatment. In summary, it appears that CD28 and its ligands B7-1 and B7-2 are immunostimulatory and beneficial in mediating T cell responses against RCC,

whereas CTLA-4 is immunoinhibitory and, thus, functions to the detriment of patients with RCC.

Recently, we have reported that increased tumor level expression of the T cell coinhibitor PD-L1 increases the risk of metastatic dissemination and cancer-specific death for patients with RCC [162-165]. These studies also demonstrated RCC-associated PD-L1 represents an independent predictive factor, even after adjusting for all other important clinical predictors. One *in vitro* experimental study has also shown that blocking PD-L1 facilitates immune responses against human RCC cells [166]. The implications of this research are important. First, PD-L1 may prove very useful as a prognostic marker to select high-risk patients that might benefit from adjuvant therapy. Second, PD-L1 may represent an important target for immunotherapeutic blockade, particularly when used in combination with other immunotherapeutic modalities [163]. Abrogating the inhibition of antitumoral T cell-mediated immunity through the use of PD-L1 blockade could provide a key strategy to permit the adaptive immune system to more easily detect and destroy malignant cancer cells, for RCC and many other forms of human malignancy.

Members of the TNF/TNFR family of costimulatory molecules have also been evaluated in RCC. For instance, CD70 has been shown to be aberrantly expressed by the majority of clear cell RCCs, and by a lesser proportion of other RCC subtypes [167]. Expression of the CD70 receptor, CD27, was also noted to be increased on NK cells of RCC patients undergoing IL-2 immunotherapy [168]. A strategy to target RCC with anti-CD70 antibody-drug conjugates has been proposed on the basis of this evidence [167]. Expression of CD40 is increased in both the vasculature and tumor cells of renal cell carcinoma patients [169,170], and stimulation with CD40L results in increased tumor cell Fas expression and death [169]. Similarly, in a murine RCC model, the combination of CD40 stimulation and IL-2 was found to be superior to either agent alone [171]. These data suggest that CD40 may play an important role in modulating immunotherapeutic antitumoral responses, though, to date, no clinical trials have tested this hypothesis.

### **Evidence from other Tumor Types**

Although costimulatory molecules have been extensively studied in cell lines and mouse models, much less is known about their function in actual human tumors. In this section we will focus on studies that assess costimulatory molecule expression in human tumors. We review three basic types of studies: tissue staining and flow cytometry (protein level) and reverse transcriptase polymerase chain reaction (mRNA level).

### **The B7/CD28 Family**

There are few human studies that assess the expression of the original members of the B7-family, B7-1 and B7-2, in cancer. Elevated levels of B7-2 have been found in acute myeloid leukemia [172]. Similarly, both B7-1 and B7-2 are expressed on the surface of intraperitoneal T cells isolated from cases of peritoneal carcinomatosis, particularly when

associated with ovarian carcinoma [173]. Interestingly, B7-1 and B7-2 could not be detected in several cancer cell lines or in gastric carcinoma [174,175].

Early research found PD-L1 mRNA expression was ubiquitous but that its protein could only be detected in macrophages and monocytes [55]. It was later shown that a variety of tumors aberrantly expressed PD-L1, including carcinomas of the lung, ovary, colon, bladder, breast, cervix, endometrium, gallbladder, larynx, liver, salivary gland, stomach, and thyroid [61,176]. Melanoma and T cell lymphoma also express PD-L1 though B cell lymphoma does not [61]. One group recently found expression of the PD-L1 receptor, PD-1, in a particular subset (CD4+CD25-) of tumor infiltrating lymphocytes in patients with non-Hodgkin's lymphoma [177]. The prognostic importance of PD-L1 seems to vary from tumor to tumor. For example, one study found no correlation between PD-L1 or PD-L2 and important clinicopathological variables or prognosis in patients with lung cancer [178]. This study did find more tumor-infiltrating lymphocytes and lower levels of PD-1 expression in areas where PD-L1 expression was high, however. Contrarily, PD-L1 and PD-L2 appear to clearly indicate a poor prognosis for patients with esophageal cancer [179]. These conflicting data may indicate that the immune system assumes divergent levels of importance in cancer control for different organ sites and histologic tumor types.

Cellular expression of ICOSL has been noted in acute myeloid leukemia patients and was correlated with worse survival [172]. ICOSL expression has also been found on malignant cells and the tumor vasculature of brain cancer specimens [180]. ICOSL expression could not be found in lung cancer [178].

Abnormally elevated levels of B7-H3 expression were seen in 59% of gastric carcinomas and 100% of gastric adenomas in one series [181]. Importantly, B7-H3 expression was associated with improved survival from these stomach tumors indicating that high levels of this molecule may help the immune system to clear cancer cells. A flow cytometry study of the bone marrow aspirates of 15 patients with neuroblastoma found B7-H3 to be a specific tumor marker [182]. It appears that B7-H3 might inhibit natural killer cell-mediated lysis of neuroblastoma cells by interacting with an undefined receptor on the surface of NK cells [182].

B7-H4 expression was studied by immunohistochemistry and was found to be positive in ovarian and lung cancers and negative in melanoma [183]. Also, the tumor infiltrating lymphocytes in lung cancer did not express B7-H4. In another study of ovarian tumors, B7-H4 was detected in all primary serous, endometrioid and clear cell carcinomas and in all metastatic serous and endometrioid carcinomas [184]. Only focal B7-H4 staining was seen in serous cystadenomas and serous tumors of low malignant potential. This suggests that B7-H4 is a marker of worsening malignant potential for ovarian tumors. A large study of breast cancers also found that B7-H4 was present in more than 95% of tissue samples, though its expression was independent of tumor grade or stage [185]. Very interesting recent results suggest that B7-H4 may be found on a fraction of tumor infiltrating macrophages and that these

macrophages have immunosuppressive activity [186]. The evidence clearly suggests that B7-H4 is a potent negative regulator of the anti-tumor immune response.

### The TNF/TNFR Family

4-1BB expression has been evaluated in various tumors. In normal lung tissue 4-1BB is expressed in basal epithelial cells and in the normal pulmonary microvasculature whereas in cancer it is found in tumor infiltrating T lymphocytes and in the tumor microvasculature [187]. One immunohistochemical study has demonstrated similar findings; specifically, that 4-1BB is strongly expressed on the endothelial cell layer of tumor-associated blood vessels in a variety of tumor types but not in healthy tissue [188]. Gene expression analysis has also demonstrated increased expression of 4-1BB in hepatocellular carcinoma but not in the normal liver [189]. Using double staining techniques, 4-1BB was localized to activated T cells, of both the CD4+ and CD8+ subtypes, but not to tumor cells. Similar results have been shown for mantle cell lymphoma [190].

A 1995 paper evaluated OX40 expression in an array of non-malignant and neoplastic tissues and, interestingly, increased OX40 expression was only detected in non-Nodgkin's lymphoma [191]. A series of studies in adult T cell leukemia have demonstrated that leukemic cells from most of these patients constitutively express OX40 [192]. Furthermore, it appears that loss of OX40 expression in cutaneous lymphomas could be a marker of increased risk of a second lymphoid malignancy [193]. This clearly has major implications. It has been suggested that adult T cell leukemia cells may escape immune-mediated apoptotic destruction through OX40 signaling and that administration of anti-OX40 monoclonal antibody may inhibit engraftment of these leukemic cells when they are injected into immunodeficient mice [194]. Clearly, OX40 is an interesting target for experimental immunotherapy [195].

Few studies have assessed the expression of CD40 or CD40L in human tumor samples. CD40 appears to be ubiquitously expressed in ovarian carcinomas and is also found in cervical cancers and their related human papillomavirus lesions [196,197]. It was also shown that CD40 affected tumor cell growth, apoptosis and immune recognition [197]. In human liver tumors, CD40 is variably expressed and appears to be induced by pro-inflammatory cytokines such as TNF $\alpha$  and IFN $\gamma$  [198]. Other studies have shown increased expression of CD40L on lymphocytes invading colon cancer and bladder cancer [199,200]. CD40L may predict a better outcome in patients with bladder cancer.

### A MODEL FOR IMMUNE DYSFUNCTION IN CANCER

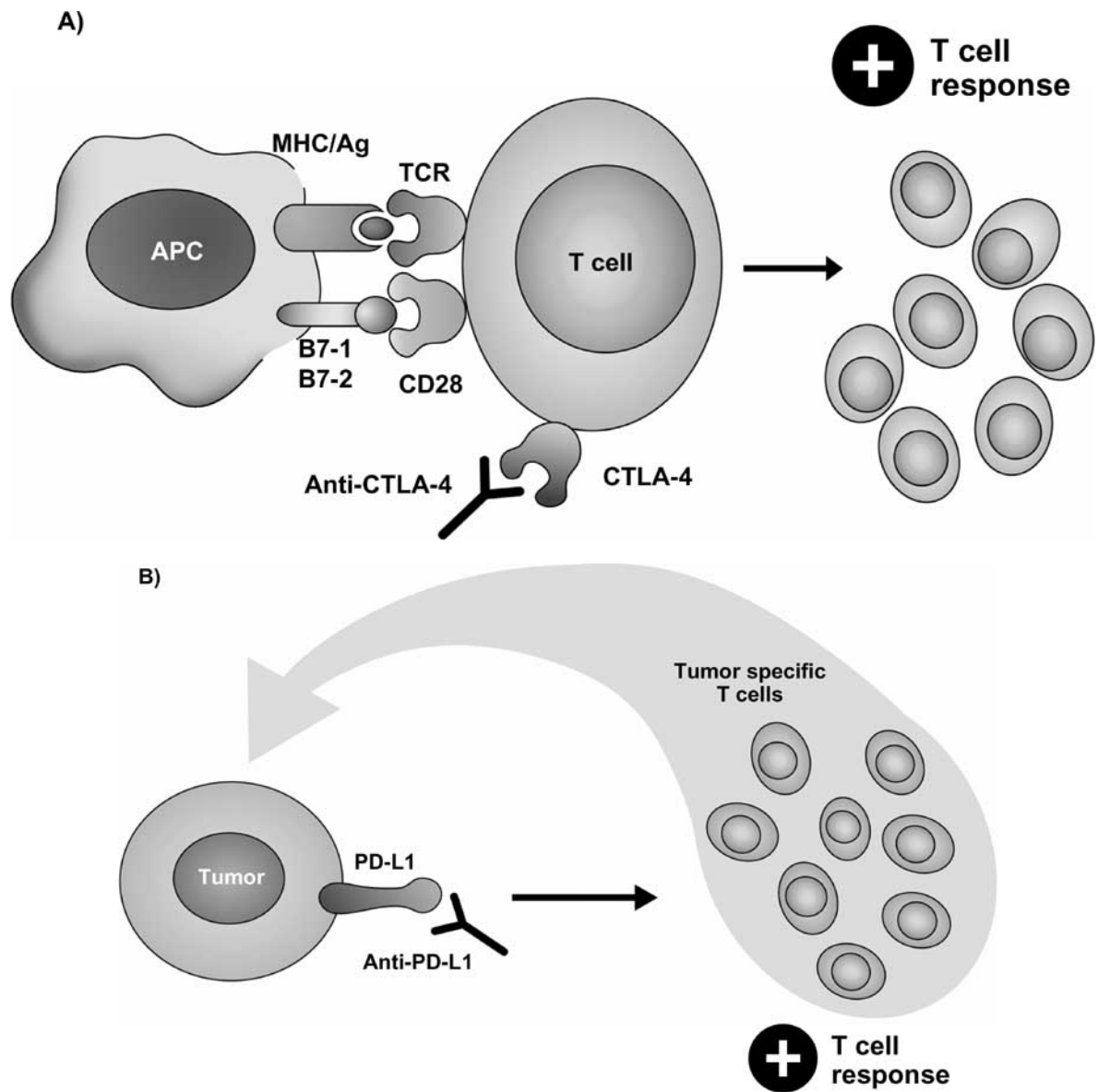
The previous sections of this review were intended to impress upon the reader what we believe to be three basic principles. Firstly, the immune system is an important defense mechanism against cancer and is therefore a worthy target for cancer therapy. Second, activating the central effector cell of adaptive immune function—the T cell—is not a serendipitous process. Rather, T cell activation requires

a highly orchestrated sequence of specific steps that can be preempted and/or disrupted by any number of critical events. Third, multiple limbs of the immune system may be impaired in cancer, and each limb is interrelated with the others. In this section, we propose a pathophysiologic paradigm that unites these three aspects into one model of tumor immunology.

The presence of distinct costimulatory and coinhibitory molecules provides a mechanism for the three prongs of immunoediting previously described. If sufficient costimulation is provided in the presence of adequate tumor-associated antigenic stimulation, the immune system will act against tumor antigen and, thus, destroy early tumors before they become fully established. Contrarily, if coinhibitory signalling dominates, the immune system will be tolerized to tumor antigens, and the tumor will be permitted to grow unfettered and unmolested by the immune system. If neither costimulatory nor coinhibitory signals dominate, the adaptive immune system may remain in a tenuous state of equilibrium, militating against tumor outgrowth with varying degrees of success.

Should a malignant cell escape early immunoediting and grow to become an established tumor, the immune system still has a chance to respond. Changes in the local biochemical environment of the tumor will result in the attraction and recruitment of naive T cells to the tumor site. These T cells once again face three potential outcomes. If the environment contains a favorable cytokine balance and positive costimulatory molecules are abundant on the tumor, the T cells will be activated and the tumor may be eliminated. On the other hand, if the tumor cells or tumor-infiltrating regulatory T cells express abundant coinhibitory molecules, then the naive T cell will become tolerized and the anti-tumor immune response will be crippled. If neither signal dominates, antitumoral T cells may mount a low level response that may slow tumor progression but does not culminate in tumor elimination.

In the particular case of RCC, evidence suggests that the major costimulatory pathway for the adaptive immune response is CD28-B7-1/2 and that the major coinhibitory pathway is PD-1/PD-L1. These may not be the principal pathways in other tumors and other cosignalling molecules may also play an important role. Nevertheless, it is clear that a T cell can be coaxed either into an activated or dormant state through manipulations of the intratumoral cytokine environment, by altering the balance of costimulatory and coinhibitory signalling, and by stimulating or inhibiting inhibitory immune cells (such as T<sub>reg</sub> cells) that act to impair regional antitumoral immunity. For patients with high risk RCC, we envision a future in which combination immunotherapeutic approaches will be required to improve treatment outcome. Such combination immunotherapies could be comprised of cytokine treatment with IFN $\alpha$  and/or IL-2, upregulation of B7-1 / B7-2 costimulation, abrogation of coinhibition through blockade of PD-L1 or CTLA-4 (see Fig. 3), and depletion of inhibitory regulatory T cells. Such immunotherapies will be given in combination with other modalities of treatment such as surgery or chemotherapy in order to optimize outcome. Thus, it is only through these multiple avenues of attack that the elimination of cancer will become a reality for patients suffering from advanced RCC.



**Fig. (3).** Potential strategies for therapeutic targeting of coinhibitory molecules. **A)** T cell CTLA-4 blockade. **B)** Tumor PD-L1 blockade.

#### ABBREVIATIONS

APC	=	Antigen presenting cell
CD	=	Cluster of differentiation
DC	=	Dendritic cell
IFN	=	Interferon
IL	=	Interleukin
MHC	=	Major histocompatibility complex
NK	=	Natural killer cell
RCC	=	Renal cell carcinoma
SMAC	=	Supramolecular activation complex
T <sub>C</sub>	=	Cytotoxic T lymphocyte
TCR	=	T cell receptor
T <sub>H</sub>	=	Helper T lymphocyte

TNF = Tumor necrosis factor

T<sub>reg</sub> = Regulatory T lymphocyte

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Received: June 02, 2006

Revised: August 08, 2006

Accepted: August 10, 2006