Immunobiology of Cancer Therapies Targeting CD137 and B7-H1/PD-1 Cosignal Pathways

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Contents

Abstract Cancer immunotherapy is finally entering a new era with manipulation of cosignaling pathways as a therapeutic approach, for which the principle was proved nearly two decades ago. In addition to CTLA-4, CD137 and B7-H1/PD-1 pathways are two new targets in the stage. CD137 pathway is costimulatory and its agonistic antibody delivers potent signal to drive T cell growth and activation. On the other hand, blockade of B7-H1/PD-1 pathway with antagonistic antibody has shown to

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protect ongoing T cell responses from impairment by immune evasion mechanism in cancer microenvironment. With these tools in hand, a mechanism-based design of combined immunotherapy with high efficacy is becoming a reality.

Abbreviations

APCs	Antigen presenting cells
DCs	Dendritic cells
HSV	Herpes simplex virus
IDO	Indoleamine-2,3-dioxygenase
LCMV	Lymphocytic choriomeningitis virus
mAb	Monoclonal antibody
MDCs	Myeloid DCs
MHC	Major histocompatibility complex
NK	Natural killer
OVA	Ovalbumin
TCR	T cell receptor
TDLN	Tumor-draining lymph node
TIL	Tumor infiltrating T lymphocytes
TNF	Tumor necrosis factor

1 Introduction

T-lymphocytes play a pivotal role in the control of cancer progression. The inherent genetic instability in tumor cells results in the expression of aberrant antigenic epitopes or the overexpression of normally repressed genes that could be recognized by T cells of the host immune system (Pardoll 2003). However, the engagement of T-cell receptors (TCRs) on T cells by the antigenic peptide/major histocompatibility complex (MHC) on the surface of antigen-presenting cells is often not sufficient to drive the activation of naive T cells leading to optimal immune responses (Lenschow et al. 1996; Chen et al. 1994). Productive T-cell activation requires a second antigen-independent cosignal, the "costimulatory signal" provided by the interaction of accessory surface molecules between T cells and antigen presenting cells (APCs). In the absence of costimulation, TCR-mediated activation of T cells resulted in antigen-specific unresponsiveness (termed T-cell anergy), rendering the T cells unable to respond to subsequent exposure to antigen (Schwartz 2003; Harding et al. 1992). On the other hand, activated T cells are under tight control by different sets of cell surface molecules through "co-inhibitory signal" to attenuate T cell responses. These co-inhibitory signals are inducible or strengthen in responding to

activation and has been shown to be a very powerful mechanism in negative regulation of T cell-mediated immune responses (Chen 2004).

CD28 is the first identified costimulatory receptor constitutively expressed on naïve T cells. Upon binding to its ligands, B7-1 (CD80) and B7-2 (CD86) on APCs, CD28 provides a potent costimulatory signal to T cells activated through their TCRs, which enhances T-cell proliferation by induction of IL-2 transcription, expression of IL-2 receptor CD25 and also confers critical survival signals to T cells through the Bcl-XL pathway. Once T cells are activated, a closely related molecule, cytotoxic T-lymphocyte antigen-4 (CTLA-4) (CD152), is induced to express on the surface of activated T cells. CTLA-4 has higher affinity than CD28 for the same ligands but appears to inhibit IL-2 production, IL-2 receptor expression, and cell cycle progression, which attenuate the immune responses and prevent autoimmune diseases (Carreno and Collins 2002; Sharpe and Freeman 2002). At present, a broad array of proteins has been identified to be involved in T-cell costimulation. The specific binding between the paired costimulatory ligands and receptors enhance or inhibit T cell responses by costimulatory signaling or coinhibitory signaling through TCRs. These cosignaling interactions form a delicate network to regulate and control initiation, expansion, development of effector and memory T cell responses as well as T cell homeostasis at multiple stages of the immune responses (Chen 2004; Wang and Chen 2004).

In the last two decades, the identification of tumor-associated antigens led to development of a variety of vaccine strategies for tumor immunotherapy, including peptides, proteins, whole cells, recombinant viral vectors, and antigen-pulsed dendritic cells, aiming to parallel the successes achieved in developing vaccines for infectious diseases. However, the attempts to target human cancers have been significantly less successful than was initially envisaged possible. In some instances, failure of consistent clinical responses including tumor regression has occurred despite impressive immunologic responses, particularly those elicited using anchor-modified epitopes as immunogens. The adoptive transfer of antigen-specific T-cells has also showed limited success in patients with melanoma and renal cell carcinoma (Morgan et al. 2006). These results offer evidences that generation of a large *in vivo* population of tumor-reactive $CD8^+$ T cells is not singularly sufficient to mediate clinically significant tumor regression. This conclusion is not so surprised because the challenges of delivering effective vaccines or immunotherapies for tumor are aligned much more closely with those associated with therapy of chronic or even ongoing infections than more acute infections, in which the majority of the successes have come with prophylactic vaccination strategies. Once the antigen-specific T cells are fully activated to become effector cells, most of them are dead of apoptosis and less develop into functional memory T cells compared to acute viral infection. Persistent antigen stimulation result in corruption of effector T cell functions such as in chronic viral infections or tumor-bearing state. Ex vivo analysis of tumor infiltrating lymphocytes (TILs) has generally demonstrated a dysfunctional state (Lee et al. 1999), which can be reversed upon culture *in vitro* (Radoja et al. 2001). It is obvious that the long-term maintenance of potent T-cells represents a significant challenge in patients with

established or recurrent tumors. Fortunately, the positive and negative costimulatory pathways have been showed to play critical roles in regulating the survival and functional corruptions (including anergy, tolerance, exhaustion, and dysfunction) of effector T cell in the settings of persistent antigen stimulation, such as chronic viral infections and the tumor-bearing state. In this context, CD137 and B7-H1/PD-1 cosignaling pathways are most promising candidates for manipulation to achieve long-term potent anti-tumor T cell responses in patients.

2 The Costimulatory CD137 Signaling Pathway

2.1 The Expression of CD137L and CD137

CD137 (4-1BB, ILA, TNFRFS9) belongs to the tumor necrosis factor (TNF) receptor superfamily and is inducibly expressed on T cells following stimulation through the TCR complex (Pollok et al. 1993). With soluble antigens, such as superantigens or ovalbumin (OVA) delivered with lipopolysaccharide (LPS), CD137 is expressed only transiently on the T cells *in vivo* (Takahashi et al. 1999). However, CD137 expression can be prolonged with persistent antigen stimulation, such as cardiac allograft rejection, adenovirus delivered antigen, persistent herpes simplex virus (HSV)-1 infection, or severe influenza infection (Tan et al. 2000; Seo et al. 2003; Lin et al. 2009). Thus, the effects of CD137 on activated T cells may depend in part on its expression pattern in the particular model studied. In addition to antigen stimulation, the cytokines of interleukin 2 (IL-2) and IL-15 can induce expression of CD137 on memory but not naive $CD8^+$ T cells *in vitro*, which may contribute to memory T cell survival after antigen clearance (Pulle et al. 2006; Sabbagh et al. 2007). Both $CD8^+$ and $CD4^+$ T cells, including Th1 and Th2 cells, can be induced to express CD137; however, at least in some circumstances, $CD8^+$ T cells can upregulate CD137 more rapidly and to higher levels than $CD4^+$ T cells (Wen et al. 2002; Futagawa et al. 2002). In addition to its well-established role as an inducible costimulatory receptor on T cells, CD137 was also expressed on activated natural killer (NK), dendritic cells (DCs), hepatoma cells and blood vessels from individuals with malignant tumors (Futagawa et al. 2002; Broll et al. 2001; Melero et al. 1998a; Schwarz et al. 1995).

CD137L, a member of TNF superfamily, was found to be expressed following stimulation on professional APCs including DCs and macrophages as well as activated B cells (Alderson et al. 1994; Pollok et al. 1994), and is also expressed on myeloid progenitors and hematopoietic stem cells (Lee et al. 2008; Jiang et al. 2008). CD137L expression appears to be tightly regulated *in vivo*, such that its expression during an ongoing immune response *in vivo* is difficult to be detected at the protein level (Lin et al. 2009). However, during chronic and inflammatory conditions CD137L is more readily detectable at the mRNA or protein level (Tan et al. 2000; Lin et al. 2009; Mack et al. 2008). The low and transient level of

CD137L expression has made it difficult to study the immediate effects of CD137L binding to CD137 *in vivo*.

When coupled with a strong signal through the TCR, engagement of CD137 can induce IL-2 production of T cells independent of CD28 ligation. Early studies demonstrated that ligation of CD137 by either cell surface CD137L or specific antibodies provide a costimulatory signal to T cells, including both $CD4^+$ and $CD8^+$ T cells, enhancing proliferation, cytokine production, and particularly survival (Takahashi et al. 1999; Schwarz et al. 1995; Alderson et al. 1994; Shuford et al. 1997; Hurtado et al. 1997). The costimulatory signal has been shown to be more potent for $CD8^+$ T cells than $CD4^+$ T cells (Shuford et al. 1997). Later studies point to the role of CD137 engagement in augmenting rather than initiating T-cell response and in sustaining their effector functions.

CD137 engagement on DCs using antibodies or transfected ligand enhances their production of inflammatory cytokines, including IL-12 (p40/70) and IL-6, and enhances their ability to activate T cells (Futagawa et al. 2002; Wilcox et al. 2002). However, the relative importance of CD137 signaling in APCs versus T cells during an ongoing immune response is unknown. Since anti-CD137 induced enhancement of T-cell expansion *in vivo* appears to act primarily through CD137 on the T cells rather than on the APCs (Sabbagh et al. 2008). Similarly, anti-CD137-induced expansion of adoptively-transferred memory T cells required CD137 on the T cells but not in the host (Zhu et al. 2007), arguing for direct effects of CD137 in the T cells.

2.2 Role of CD137 Cosignaling in Effector/Memory T Cells

The up-regulation of CD137 on antigen-experienced T cells suggests that CD137 cosignaling may target these primed T cells differentially, influencing those T cells preferentially with highest avidity receptors. When wild-type or CD137L-deficient mice were infected intraperitoneally with influenza A/X31, the primary expansion and contraction of $CD8^+$ T cells was indistinguishable over the first 2 weeks of the response. However, there was a two- to threefold defect in the number of $CD8^+$ T cells persisting at 3–5 weeks after infection, and a two- to threefold decrease in the recall response to influenza A/PR8 in the CD137L-deficient mice (Bertram et al. 2002). This observation revealed a role for CD137L in controlling influenza specific effector-memory T-cell numbers late in the primary response. In an adoptive transfer experiment, TCR-transgenic T cells were cultured with OVA antigen peptide followed by human IL-15 to produce the cells with the surface phenotype of central-memory T cells ($CD62L^{high}$ $CD44^{high}$ IL-$7R^+$ $CCR7^+$ $CD69^+$) (Manjunath et al. 2001). Transfer of these *in vitro*-derived "memory phenotype" cells into otherwise unmanipulated naive-wild-type or CD137L-deficient mice showed that the absence of CD137L in the host resulted in a two- to threefold decrease in the number of adoptively transferred T cells recovered in the spleen and bone marrow compared with wild-type mice by 3 weeks post transfer under conditions where the

rate of cell division (about one or two divisions in 3 weeks) was indistinguishable in the mice (Pulle et al. 2006). Taken together, the CD137/CD137L interaction may play a role in survival of effector-memory $CD8^+$ T cell after antigen has been cleared.

Our recent study found that systemic administration of anti-CD137 antibodies induced expansion of $CD4^+$ and $CD8^+$ T cells with memory but not naïve phenotype in mice (Zhu et al. 2007). The T cell activation and proliferation is antigen independent. CD137 is required on the T cells and is dispensable in the host for anti-CD137 to expand memory T cells. With systemic antibody treatment, both $CD4^+$ and $CD8^+$ memory T cells were expanded, and this expansion was due to increased division as measured by bromodeoxyuridine incorporation. In contrast, CD137L deficiency influenced the $CD8^+$ but not the $CD4^+$ memory T-cell pool largely through effects on survival rather than on cell division (Pulle et al. 2006). It is possible that the supraphysiological anti-CD137 or overexpressed CD137L signal is sufficient to drive T-cell proliferation, whereas the endogenous level of CD137L in the host may not be sufficient for this effect. In summary, data from both knockout mice as well as from systemic treatment of unimmunized mice with stimulatory anti-CD137 antibodies support a role for CD137 on memory T cells receiving signals from CD137L in the host to maintain $CD8^+$ T-cell memory.

3 Strategies to Augment Tumor Immunity by Stimulating CD137

Ligation of costimulatory receptor CD137 by either its ligand or agonistic antibodies has been shown to provide a potent costimulatory signal, which is more potent for $CD8^+$ T cells than for $CD4^+$ T cells. Particularly, CD137 is specifically expressed on antigen-activated T cells and provides a potent costimulatory signal for enhancing the functions of effector/memory T cells and for maintaining the T cells survival. These results raise great interest in manipulation of the CD137 pathway as a therapeutic target for cancer therapy.

3.1 Tumor Therapy with Agonist Anti-CD137 Antibody

In an early study, agonist anti-CD137 mAb was shown to induce regression of established tumors including the poor immunogenic Ag104A sarcoma and the highly tumorigenic p815 mastocytoma in mouse models (Melero et al. 1997). The anti-tumor effect required both $CD4^+$ and $CD8^+$ T cells and was accompanied by marked augmentation of tumor-specific CTL activity. Subsequent studies showed that agonist anti-CD137 mAbs induced the complete regression of many types of established tumors induced by transplantable syngeneic mouse tumor lines (May et al. 2002; Kim et al. 2001; Lynch 2008).

The mechanisms of CD137 mAb-mediated tumor regression are yet to be elucidated. Several possible mechanisms include the breaking of immunological ignorance, prevention of T cell tolerance/anergy and deletion, and are largely dependent on the models employed. In an established C3 tumor model, CTLs against a model tumor antigen, human papillomavirus E7 oncoprotein, are not anergic or deleted, but remain naïve. Immunological ignorance of specific CTLs appears to prevent anti–CD137 mAb from activating tumor immunity, since anti–CD137 mAb itself neither activates tumor-specific CTLs nor induces the regression of established C3 tumors. Similar observations were made in the TC-1 lung carcinoma and B16-F10 melanoma models. Immunization with E7 tumor peptide in the presence of adjuvant stimulated E7-specific $CD8^+$ CTL, leading to elimination of T cell ignorance, albeit is still insufficient to regress the established C3 tumor. In combination with anti-CD137 mAb, a high level of E7-specific CTLs was elicited, leading to the complete regression of established C3 tumors *in vivo*. These studies indicate that cancer cells may not be able to initiate antigen-specific CTL response and initial trigger of such responses is critical. Similarly, treatment of tumor-bearing mice with Flt3L (a cytokine that promotes the generation of large numbers of DCs *in vivo*) resulted in the generation of effective $CD8^+$ T-cell-mediated immune responses by enhancing the efficiency of antigen presentation to T cells. There was a clear cooperative effect when Flt3L and anti-CD137 treatments were combined in tumor-bearing mice (Miller et al. 2002). Treatment of mice that have been immunized with either GM-CSF-secreting tumor cells or a DC-based vaccine with CD137 mAb also results in augmentation of the anti-tumor immune responses (Li et al. 2007; Ito et al. 2004). Collectively, these results suggest that selective stimulation of antigen-specific T cells is prerequisite for CD137 agonists to operate. Interestingly, several reports in rodent models have also shown synergistic effects of agonistic anti-CD137 mAb in combination with anti-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and CD40 mAbs (Uno et al. 2006), with intratumoral introduction of the IL-12 gene (Xu et al. 2005), or with chemotherapy or radiotherapy (McMillin et al. 2006; Shi and Siemann 2006). These observations provide important information for developing combination studies in the clinic.

A somewhat unusual feature accompanied with anti-CD137 mAb therapy is diminished pathology in autoimmune disease models. Sun and colleagues showed that the same agonist anti-CD137 antibody used to promote anti-tumor immunity resulted in ameliorating both the incidence and severity of experimental autoimmune encephalomyelitis (EAE) (Sun et al. 2002a). Many other studies have confirmed the beneficial effects of anti-CD137 mAb in various autoimmune disease models including rheumatoid arthritis (Seo et al. 2004), and systemic lupus erythematosis (Vinay et al. 2006). In a transplantation model, anti-CD137 was also shown to inhibit rejection of intestinal allografts in mice (Wang et al. 2003a). Although a complete understanding of these differential effects is lacking, the promotion of regulatory T cell development and activity is believed to play a role. Additional potential explanations for immune suppression in some settings include the apparent ability to delete $CD4^+$ T cells, retard B-cell function, and upregulation of

indoleamine-2,3-dioxygenase (IDO) and IFN-γ (Sun et al. 2002a, 2002b; Seo et al. 2004; Vinay et al. 2006). Therefore, anti-CD137 mAb represents a unique immunotherapeutic agent that could be applied to both immune potentiating for tumor immunity and immune suppressive for certain autoimmune diseases.

3.2 Whole Cell Vaccines with Capacity to Stimulate CD137

Introduction of costimulatory molecules into tumor cells to improve its ability as better APCs has become a common therapeutic vaccine in tumor immunotherapy. Transfection of tumor cells to express CD137L showed to enhance immunogenicity of murine P815 mastocytoma or AG104A sarcoma lines by developing a strong CTL response, which rejected these tumors (Melero et al. 1998b). Although CD137 is an independent costimulator, optimal effect of CD137L in CTL stimulation may require B7-CD28 interaction since blockade of this interaction by antibodies down-regulated the expression of CD137 on T cells and decreased CTL activity. Furthermore, co-expression of CD137L and B7-1 in the poorly immunogenic AG104A sarcoma enhanced the induction of effector CTL and the rejection of the wild-type tumor while neither CD137L nor B7-1 single transfectants were effective (Melero et al. 1998b). In a later study using the A20, a B-cell lymphoma cell line, a similar result was obtained (Guinn et al. 1999). These results suggest that a synergistic effect between the CD137 and the CD28 co-stimulatory pathways in the anti-tumor immune responses. Although CD137 can function independently of CD28 to deliver a co-mitogenic signal for T cell proliferation and IL-2 production (DeBenedette et al. 1997), B7-CD28 costimulatory signaling is required for the optimal expression of CD137 on T cells, which in return further promote the anti-tumor functions of the activated T cells.

In order to deliver CD137L to tumor for tumor therapy, a replication-defective adenovirus expressing CD137L gene was constructed. In a syngeneic mouse model of liver tumor metastasis induced by intrahepatic injection of the poorly immunogenic MCA26 colon cancer cells, various combinations of replication-defective adenoviruses expressing IL-12 and CD137L cDNA were injected into the established liver tumor. The long-term survival rate of mice treated with the combination of IL-12 and CD137L were significantly improved over that of animals in the control groups. *In vivo* depletion of NK cells or $CD8^+$ T cells completely abolished the long-term survival advantage of the IL-12 plus CD137L-treated animals (Martinet et al. 2000). When combined with IL-12 gene transfer, systemic administration of the Ig-CD137L fusion protein can generate a better antitumor response than local gene delivery. In this combination therapy, the Ig-CD137L is as potent as the agonistic anti-CD137 antibody for the treatment of hepatic MCA26 colon carcinoma, resulting in 50% complete tumor regression and long-term survival. In long-term surviving mice, both treatment modalities induced persistent tumor-specific CTL activity (Xu et al. 2005).

It would appear that the transfection of CD137L cDNA into tumor cells as a whole cell tumor vaccine may not be as effective as anti-CD137 mAb. In the murine sarcoma line Ag104, the tumor cells which express CD137L had no therapeutic activity unless they were also transfected with B7-1 (Melero et al. 1998b). However, the injection of anti-CD137 mAb into mice with the same tumor (Ag104) caused tumor destruction (Melero et al. 1997). In order to create a vaccine that stimulate the immune system as a monoclonal antibody does, Ye and colleagues constructed a vector encoding cell-bound single-chain Fv fragments from a hybridoma secreting CD137 mAb, and transfected to express this gene into K1735 melanoma cells which expressed low levels of MHC class I molecules and were poorly immunogenicity. Mice vaccinated with modified tumor cells rejected established wild-type K1735 tumors growing as subcutaneous nodules or in the lung (Ye et al. 2002).

3.3 Adoptive Transfer of T Cells with CD137 Costimulation

Adoptive transfer of tumor-reactive T cells represents an effective immunotherapeutic strategy for cancer treatment. Clinical trials have demonstrated beneficial effects of adoptive immunotherapy in malignant melanoma (Dudley et al. 2002; Yee et al. 2002), renal cell carcinoma (Kawai et al. 2003), EBV-associated nasopharyngeal carcinoma (Straathof et al. 2005), Hodgkin's Disease (Bollard et al. 2004) and glioma (Tsuboi et al. 2003) with ex vivo expanded CTLs. Efficacy of T-cell adoptive transfer may be improved through optimization of *in vitro* expansion, characterization of effector populations, and/or by enhancing the function and survival of transferred CTLs to facilitate establishment of immunologic memory.

Several studies have examined the possibility of using CD137 costimulation for the generation of tumor-reactive T cells for adoptive immunotherapy. Addition of an agonistic anti-CD137 mAb to *in vitro* cultures of tumor-draining lymph node (TDLN) cells with anti-CD3/anti-CD28 antibodies enhanced expansion, type 1 cytokine production, and survival of T cells. When anti-CD3/anti-CD28/anti-CD137-expanded TDLN cells were adoptively transferred into MCA 205 tumor-bearing mice, significantly fewer metastatic lesions and prolonged survival of mice were observed compared with TDLN cells stimulated without anti-CD137 (Li et al. 2003). Strome et al. (2000) isolated T cells from the TDLN of mice bearing disseminated micrometastasis of a poorly immunogenic, MHC class I-negative A9P squamous cell carcinoma. The T cells expended with combination of anti-CD3/anti-CD28/anti-CD137 were more effective than those activated by anti-CD3 alone or anti-CD3/anti-CD28 in mediating antitumor reactivity.

Currently, a majority of investigators generated T cells for clinical trials of adoptive immunotherapy by repetitive TCR-based stimulation of peripheral blood lymphocytes using various APCs. The CD28 costimulation is a commonly used approach for expanding T cells since B7/CD28 pathway is widely considered an important costimulatory pathway for TCR activation. However, bead-based

anti-CD3/CD28 artificial APCs (aAPCs) induced brisk expansion of $CD4^+$ populations but not $CD8^+$ T cells (Deeths et al. 1999; Laux et al. 2000), demonstrating that the CD28 costimulation may not be sufficient for expansion of $CD8^+$ CTL. Maus et al. (2002) demonstrated that the incorporation of CD137L into an aAPC greatly augmented the capacity for Ag-specific expansion of $CD8^+$ T cells *ex vivo*. Zhang et al. (2007) directly compared the efficacy of CD28 vs. CD137 signaling in the expansion of $CD8^+$ CTL, and showed that anti-CD3/CD137L aAPCs preferentially expand memory $CD8^+$ T cells, resulting in an increased frequency of cells responding to viral recall antigens in the expanded cultures from healthy donors, whereas anti-CD3/anti-CD28 aAPCs preferentially expand naïve $CD8^+$ cells and therefore do not enrich for viral-specific CTL. The CTL expanded using CD137 costimulation mediate enhanced cytolytic capacity compared with using CD28 costimulation. For more effectively expanding tumor-specific CTL, the recombinant replication-defective adenovirus and HSV amplicons encoding CD137L were constructed and used to convert autologous monocytes or tumor cells into efficient APC (Serghides et al. 2005; Yi et al. 2007). These systems provide theoretical means to selectively expand tumor-specific effector populations without the need for pre-sorting for tumor-reactive T cells.

As an alternative to deliver the CD137 signal, Stephan and colleagues (Stephan et al. 2007) recently employed a genetic approach to constitutively co-express CD80 and CD137L in primary human cytomegalovirus (CMV)-specific T cells and prostate-specific membrane antigen (PSMA)-targeted T cells, substituting for the lack of these ligands on APCs. The T cells expressing CD80 and CD137L vigorously respond to tumor cells lacking costimulatory ligands and provoked potent rejection of large, systemic tumors in immunodeficient mice. These findings obtained in a very challenging tumor model, underscore the remarkable biological activity and potency of constitutive, high-level expression of costimulatory ligands on T cells.

4 The Coinhibitory B7-H1/PD-1 Signaling Pathway

4.1 Expression of B7-H1, B7-DC and PD-1

B7-H1 (CD274) (Dong et al. 1999) and B7-DC (CD273) (Tseng et al. 2001) were initially identified as a potential costimulatory molecule that could stimulate T cell responses in the presence of TCR signaling. While the overall expression of B7-H1 and B7-DC transcripts is similarly found in various lymphoid and nonlymphoid tissues (Dong et al. 1999; Freeman et al. 2000; Latchman et al. 2001; Tamura et al. 2001), the expression profiles of cell surface proteins are quite distinct. The expression of B7-H1 protein, although virtually absent in normal tissues except macrophage and dendritic cell-like cells, could be induced in a variety of tissues and cell types, such as DCs, macrophages, B cells, T cells, NK cells, and bone

marrow-derived mast cells, epithelial cells, muscle cells, trophoblast, endothelial cells and various tumor cells (Dong et al. 1999; Tamura et al. 2001). On the contrary, cell surface B7-DC was mainly detected on several types of myeloid cells including DCs and macrophages (Tseng et al. 2001). Cell surface expression of both B7-H1 and B7-DC could be up-regulated upon activation or IFN-γ treatment of human monocytes and DCs (Tseng et al. 2001; Dong et al. 2002).

PD-1 is a distant homologue of CTLA-4 molecule, which was originally identified as a gene that was highly expressed by cell lines undergoing programmed cell death (Ishida et al. 1992). PD-1 is not detectable on naive T cells but its expression goes up in T cells, B cells, and myeloid cells after activation (Freeman et al. 2000; Agata et al. 1996). PD-1 expression is also upregulated on purified human T cells by cytokines using the common gamma chain including IL-2, IL-7, IL-15, and IL-21 in the absence of TCR ligation (Kinter et al. 2008). PD-1 is retained in an intracellular compartment of freshly isolated regulatory T cells, but is translocated to the cell surface after TCR stimulation (Raimondi et al. 2006). The expression of PD-1 is particularly high on the surface of functionally exhausted $CD8^+$ effector T cells during persistent viral infections in both mice and humans (Barber et al. 2006; Day et al. 2006).

4.2 Complex Interactions Among B7-H1, B7-DC, B7-1, PD-1, and Possible Additional Binding Partners

Both B7-H1 and B7-DC were found to bind PD-1 and, therefore, were renamed as PD ligand 1 (PD-L1) and PD ligand 2 (PD-L2), respectively, to emphasize PD-1 as a receptor (Freeman et al. 2000; Latchman et al. 2001). This nomenclature, however, undermines the complex costimulatory interactions within this pathway because subsequent studies demonstrated that B7-H1 could suppress T cell responses by interacting with another independent receptor B7-1 (Butte et al. 2007, 2008). Furthermore, B7-H1 was shown to be a receptor and could utilize PD-1 as a ligand to deliver an anti-apoptotic signal (Azuma et al. 2008).

The predominant role of PD-1 is inhibitory for immune responses, and this notion is supported by the phenotypes of lymphoproliferative/autoimmune diseases in PD-1-deficient mice (Okazaki and Honjo 2006). There is ample evidence that the major ligand for the suppressive function of PD-1 *in vivo* appears to be B7-H1 because results obtained from PD-1 or B7-H1 deficient mice as well as blocking antibodies against PD-1 or B7-H1 are often similar (Nishimura et al. 1999, 2001). However, B7-H1 deficient mice do not develop autoimmune diseases, albeit mild to moderate levels of $CD8^+$ T cell accumulation are common in peripheral organs (Dong et al. 2004). In T cell culture systems, studies reveal either positive or negative function of B7-H1 and B7-DC in T cell growth and cytokine production, highlighting a lack of reliable *in vitro* models for the prediction of their functions *in vivo*. Possible interpretations for these somewhat confusing data are either

additional receptor(s) for B7-H1 and B7-DC or possible receptor functions of these so-called "ligand" molecules. B7-H1 was recently shown to mediate suppressive functions through B7-1 on T cells (Butte et al. 2007). Our studies using structural biology and site-directed mutagenesis approaches have led to the characterization of B7-H1 and B7-DC mutants with abolished PD-1 binding capacity (Wang et al. 2003b). Interestingly, several such mutants are still able to costimulate proliferation and cytokine production of T cells from normal or even PD-$1^{-/-}$ mice at a comparable level to wild type B7-H1 and B7-DC. The costimulation of B7-DC in conjunction with B7-1 for cytokine production is also shown to be PD-1 independent (Shin et al. 2003). Therefore, B7-H1 and B7-DC may costimulate T cell growth through a receptor other than PD-1 and B7-1. In addition to being a ligand, B7-H1 could also act as a receptor and utilize PD-1 as the ligand. By transfection of intracellular domain-deficient B7-H1 or PD-1 into tumor cells or T cells, respectively, cancer cells expressing truncated B7-H1 lost their resistance to lysis by tumor antigen-specific $CD8^+$ T cells. In contrast, truncated PD-1 on T cells was still able to act as a ligand for full length B7-H1 on cancer cells to deliver an anti-apoptotic signal (Azuma et al. 2008). Therefore, it is premature to conclude that the interaction between B7-H1 and PD-1 is exclusively suppressive.

4.3 B7-H1/PD-1 Interaction in the Suppression of Immune Responses

The broad distribution of B7-H1 in non-lymphoid organs and the autoimmune phenotype of PD-$1^{-/-}$ mice suggest a role of PD-1 signaling in the regulation of peripheral self-tolerance of T cells. PD-$1^{-/-}$ mice on the C57BL/6 background develop a lupus-like arthritis (Nishimura et al. 1999), while BALB/c mice develop a cardiomyopathy secondary to the production of an autoantibody directed against cardiac troponin (Nishimura et al. 2001; Okazaki et al. 2003). The autoimmunity that occurred in PD-$1^{-/-}$ mice is different from that which developed in CTLA-$4^{-/-}$ mice. The CTLA-4 deficient mice died within 3∼4 weeks of birth from massive lymphocytic infiltration and tissue destruction in critical organs (Waterhouse et al. 1995; Tivol et al. 1995), while PD-$1^{-/-}$ mice developed strain-specific autoimmunity in old age (Nishimura et al. 1999, 2001). These differences may reflect the different regulatory roles of these two negative cosignaling pathways in self-reactive T cells. CTLA-4 signaling controls the activation of self-reactive T cells, while PD-1 signaling plays critical roles in regulating the effector functions of activated self-reactive T cells in peripheral tissues. In addition, PD-1 and B7-H1 have also been shown to be involved in fetomaternal tolerance (Guleria et al. 2005), the regulation of alloimmune responses (Sandner et al. 2005), graft-versus-host disease (Blazar et al. 2003), and autoimmune disease in multiple mouse models (Wang et al. 2005; Salama et al. 2003; Ansari et al. 2003; Matsumoto et al. 2004). These results suggest

a predominant role of B7-H1 and PD-1 interactions in the establishment and/or maintenance of peripheral tolerance.

B7-H1/PD-1 interaction appears to play a critical role in regulation of exhausted virus-specific $CD8^+$ effector T cells during persistent viral infections. PD-1 is upregulated upon activation, and a functionally significant high level of expression is maintained by exhausted $CD8^+$ T cells in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) (Barber et al. 2006). *In vivo* administration of antibodies that block the interaction of B7-H1 and PD-1 restores the ability of the exhausted $CD8^+$ T cells to proliferate, secrete cytokines, kill infected targets, and decrease viral load in the animals. Similarly, PD-1 is expressed at high levels on non-functional T cells during human immunodeficiency virus (HIV) infections, and anti-PD-1 or anti-B7-H1 antibodies are able to restore their proliferation and effector functions, at least *in vitro* (Day et al. 2006; Trautmann et al. 2006). Comparable findings have been observed during chronic infections with hepatitis B and C viruses (Boni et al. 2007; Urbani et al. 2006; Penna et al. 2007), Helicobacter pylori (Das et al. 2006), and Mycobacterium tuberculosis (Jurado et al. 2008). PD-1 expression was dramatically upregulated on effector $CD8^+$ T cells in acute and chronic LCMV infection, and was then rapidly downregulated after the virus is cleared in acutely infected mice. In contrast, PD-1 expression continued to increase on virus-specific $CD8^+$ T cells in chronically infected mice, and the high level of expression was sustained (Barber et al. 2006).

5 Manipulation of B7-H1/PD-1 Pathway in Tumor Immunotherapy

5.1 B7-H1/PD-1 Pathway in the Evasion of Tumor Immunity

Our early observation that multiple human tumor lines and freshly isolated cancer cells over-express B7-H1 (Dong et al. 2002) has prompted the investigation of the potential role of B7-H1/PD-1 pathway in the regulation of tumor immunity. Many human cancers have been reported to aberrantly express B7-H1 (Dong et al. 2002; Hamanishi et al. 2007). Upregulation of B7-H1 is strongly associated with local inflammatory and immune responses because IFN-γ is found to be the most potent inducer of B7-H1 (Keir et al. 2008). Retrospective studies showed a significant correlation of intra-tumor B7-H1 expression with poor prognosis in ovarian cancer (Hamanishi et al. 2007), renal cancer (Thompson et al. 2004, 2006), pancreatic cancer (Nomi et al. 2007), breast cancer (Ghebeh et al. 2006), and bladder urothelial carcinoma (Inman et al. 2007; Nakanishi et al. 2007). In addition, 41% of TILs expressed B7-H1 in breast cancer, which was associated with a large tumor size and Her2/neu–positive status (Ghebeh et al. 2006). In renal cell cancer, a high expression level of B7-H1 on both tumor cells and TILs correlated with aggressive tumor behavior and was associated with a 4.5-fold higher risk of cancer-related death than

patients with low B7-H1 (Thompson et al. 2004). Interestingly, the expression level of B7-H1 on tumor cells was found to correlate inversely with numbers of ovarian intraepithelial $CD8^+$ T cells, the presence of which was associated with improved patient outcomes (Hamanishi et al. 2007). In contrast to B7-H1, expression of B7-DC in tumors was much less frequent, which is due to the fact that B7-DC is generally limited to myeloid cells.

To test whether over-expression of B7-H1 on tumor cells impaired anti-tumor immunity, murine immunogenic P815 cells were transfected to express B7-H1. B7-H1 expressing tumor cells were relatively resistant to *in vitro* cytolysis of tumor-specific CTL compared with control P815 cells (Iwai et al. 2002; Hirano et al. 2005) and resistant to immunotherapy of anti-CD137 mAb. But blockade with anti-B7-H1 restores the response to anti-CD137 treatment (Hirano et al. 2005). In a different model, the effect of over expression of B7-H1 on the murine squamous cell cancer cell line SCCVII resulted in diminished immune-mediated control that was restored upon B7-H1 blockade (Strome et al. 2003). Tumor outgrowth of the naturally B7-H1-expressing J558L myeloma cell line was controlled in syngeneic PD-$1^{-/-}$ mice and in wild type mice treated with anti-B7-H1 mAb (Iwai et al. 2002).

In addition to a direct effect by B7-H1 expressed on tumor cells, tumor-associated APCs can also utilize the B7-H1/PD-1 pathway to control antitumor T cell responses. Myeloid DCs (MDCs) generated from peripheral blood of ovarian cancer patients express high levels of B7-H1, which could be upregulated by tumor environmental factors IL-10 and vascular endothelial growth factor (VEGF) *in vitro*. T cells stimulated in the presence of autologous tumor MDCs and anti-B7-H1 mAb augmented T-cell effector function and led to improved control of the growth of human ovarian carcinomas inoculated in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice (Curiel et al. 2003). Plasmacytoid DCs in the TDLN of B16 melanoma express IDO, which strongly activates the suppressive activity of regulatory T cells. The suppressive activity of IDO-treated regulatory T cells required cell contact with IDO-expressing DCs and was abrogated by B7-H1 blockade (Sharma et al. 2007).

Although the outcome of B7-H1 expression on cancer microenvironment, especially on cancer cells, remains to be determined in a prospective study, ample evidence supports that aberrant over-expression of B7-H1 on tumor cells impairs antitumor immunity – resulting in the immune evasion in cancer microenvironment. The mechanisms by which tumor-associated B7-H1 might protect tumors from T-cell-mediated immune destruction have been explored by the induction of tumor-specific T cell death (Dong et al. 2002) or by making the tumor cells resistant to T-cell-mediated destruction (Hirano et al. 2005). Like the "exhausted" viral antigen-specific T cells in chronic viral infection, the majority of TILs express high level of PD-1 compared with the T cells in normal tissues and peripheral blood lymphocytes. The overwhelming majority of CD8 T cells specific for the tumor differentiation antigen MART-1/ Melan-A (hereafter, MART-1) expressed high levels of PD-1 in tumors compared with MART-specific T cells in peripheral blood in the same patients. PD-1 expression correlated with an exhausted phenotype and

impaired effector function (Ahmadzadeh et al. 2009). Two independent groups (Zhang et al. 2009; Mumprecht et al. 2009) also showed that tumor-specific CTLs express high levels of PD-1 and have impaired function in a mouse model of chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML). They confirmed not only that PD-1 expression is a marker of T-cell exhaustion, but also that B7-H1 expressed by tumor cells contributes to T-lymphocyte dysfunction. These findings suggest that the tumor microenvironment can lead to up-regulation of PD-1 on tumor-reactive T cells and contribute to impaired antitumor immune responses.

5.2 Blocking B7-H1/PD-1 Pathway in Cancer Therapy

With extensive data showing that the B7-H1/PD-1 pathway plays a critical role in evasion of tumor immunity, ironically, blockade of B7-H1 or PD-1 by mAb as single agent is often not very effective in treating established tumors induced by transplantable murine tumor lines. In several tumor models using highly immunogenic murine tumors, marginal to moderate therapeutic effects were observed (Nomi et al. 2007; Iwai et al. 2002, 2005; Strome et al. 2003; Webster et al. 2007). These findings, however, are not totally unexpected because blockade of B7-H1 or PD-1 is not expected to directly stimulate immune responses, but protect ongoing T cell responses to tumor antigens. In the majority of transplantable tumor models, rapid growth of transplanted tumors in syngeneic mice may not allow development of a significant T cell response against the tumor, and the majority of tumor antigen-specific T cells remain ignorant (Chen 1998). To support this notion, immunization of tumor-bearing mice with cancer vaccines (Webster et al. 2007) or other means to stimulate T cell response (Nomi et al. 2007; Hirano et al. 2005; Strome et al. 2003) together with blockade of anti-B7-H1 or anti-PD-1 mAb often gives dramatic synergistic effects. These observations highlight the importance of mechanism–based design of cancer therapeutics to maximize efficacy.

Two phase I clinical trials using anti-PD-1 antibodies have been completed for the treatment of patients with advanced malignancies. CT-011 is a humanized antibody against PD-1, and the ability of CT-011 to enhance the function of human tumor-specific T cells has been tested *in vitro* (Wong et al. 2007). Blockade of PD-1 with this mAb during *in vitro* stimulation with melanoma peptide increased the numbers and effector activity of tumor-specific human T cells. Both Th1 and Th2 cytokine production were increased. PD-1 blockade did not change the percentage of apoptotic antigen-specific human T cells, suggesting that the increase in number was due to increased proliferation, not decreased death. A phase I clinical trial in 17 patients with advanced hematologic malignancies showed that this antibody was well tolerated and has had clinical benefit in 33% of patients with one complete remission. Development of autoimmunity was not reported in this trial (Berger et al. 2008). In a phase I study of MDX-1106, a fully human mAb against PD-1, in patients with advanced solid cancer, 39 patients with colorectal

cancer, melanoma, prostate cancer, non-small cell lung carcinoma and renal cell carcinoma were treated with MDX-1106 from 0.3 to 10 mg/kg. Administration of MDX-1106 was safe in general and even high doses of antibody were well tolerated. Toxicities include grade 2–3 anemia, lymphopenia, colitis, and arthritis. Clinical responses include one durable complete response (CR), two partial response (PR) and two mixed responses in 39 patients (Brahmer et al. 2008).

Experimental and clinical results indicates that manipulation of B7-H1/PD-1 pathway provides a new class of agents and represents a promising new strategy for tumor therapy that might be able to synergize with other therapeutic approaches to increase efficacy of the cancer treatments.

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