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Review Article

Targeting Costimulatory Molecules to Improve Antitumor Immunity

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The full activation of T cells necessitates the concomitant activation of two signals, the engagement of T-cell receptor by peptide/major histocompatibility complex II and an additional signal delivered by costimulatory molecules. The best characterized costimulatory molecules belong to B7/CD28 and TNF/TNFR families and play crucial roles in the modulation of immune response and improvement of antitumor immunity. Unfortunately, tumors often generate an immunosuppressive microenvironment, where T-cell response is attenuated by the lack of costimulatory molecules on the surface of cancer cells. Thus, targeting costimulatory pathways represent an attractive therapeutic strategy to enhance the antitumor immunity in several human cancers. Here, latest therapeutic approaches targeting costimulatory molecules will be described.

1. Introduction

T-cell activation requires a double signal, as stated in the "two signal theory." The first signal is provided by the engagement of the T-cell receptor (TCR) by its cognate antigen, through the interaction with the peptide-major-histocompatibility complex (MHC) on antigen presenting cells (APCs). In 1987, Jenkins et al. [1] demonstrated that TCR engagement was not sufficient for a full T-cell activation. Costimulatory molecules expressed on the surface of APCs are responsible for the second signal, known as costimulatory signal. The interactions of costimulatory molecules with cognate receptors on the surface of T cells result in clonal T-cell expansion and differentiation, as well as in carrying out their effector functions [2]. For several costimulatory molecules a bidirectional signaling has been reported, because their signaling pathways are also directed toward APCs. The lack of costimulation results in a nonresponsive state of T cells, known as anergy [3]. Following the initial activation, coinhibitory molecules are induced to dampen the immune response. Complex interactions implicating both overlapping and distinct costimulatory pathways underlie the generation of the immune response; thus, the tightly regulated expression of costimulatory and coinhibitory molecules, both in time and space, is crucial to provide an efficient immune protection avoiding autoimmunity.

Costimulatory molecules belong to two major families: B7/CD28 family and tumor necrosis factor (TNF)/tumor necrosis factor receptor (TNFR) family. All molecules belonging to B7/CD28 family are members of the larger immunoglobulin superfamily and are involved in the triggering of cell-mediate immune response. Instead, the TNF/TNFR family members are involved in the later phases of T-cell activation and are induced from hours to days following the TCR engagement [2].

The presence of an efficient costimulation is crucial for improving antitumor immunity. In fact, one of the mechanisms through which tumors are able to evade immune surveillance is the lower expression of costimulatory molecules or the upregulation of coinhibitory molecules. The lack of costimulation in the tumor microenvironment could be responsible for the generation of anergic T cells and, consequently, the absence of an appropriate antitumor immune response [4].

This paper focuses on the major costimulatory pathways belonging to B7/CD28 and TNF/TNFR families, underlying

the potential of targeting these pathways in cancer immunotherapy.

2. The B7:CD28 Family

2.1. B7-1/B7-2:CD28/CTLA-4. The B7-1/B7-2:CD28/CTLA-4 pathway is the best characterized pathway of T-cell costimulation and coinhibition and symbolizes the classical way where the ligand can bind two receptors for regulating both T-cell activation and tolerance. The balance between the activating and inhibitory signals derived from the engagement of CD28 and CTLA-4, respectively, is crucial to assure protective immunity, without falling into undesired autoimmunity.

B7-1 (CD80) and B7-2 (CD86) are two ligands for both CD28 and CTLA-4. The expression of B7-1 and B7-2 is restricted to professional APCs, such as dendritic cells (DCs), macrophages, and B cells. CD28 is constitutively expressed on the surface of T cells, whereas CTLA-4 expression is induced 24–48 hours after T-cell activation, due to the action of lymphocyte cell kinase (Lck), Fyn and resting lymphocyte kinase (RLK) that phosphorylates CTLA-4, thus increasing its transport to the cell surface and preventing its internalization. CTLA-4 was shown to have higher affinity for both B7-1 and B7-2 than CD28 receptor [4, 5].

The B7-1/B7-2:CD28 pathway is the strongest costimulatory signal delivered by APCs to provide a full activation of T cells, promoting their proliferation and IL-2 secretion [4]. The intracellular signaling of B7-1/B7-2:CD28 pathway occurs through the activation of phosphatidyl-inositol-3-kinase (PI3K)/Akt/Nuclear Factor-κappa B (NF-κB) and the mitogen-activated protein kinases (MAPKs) pathway, which support cell survival, memory development, proliferation, and cytokines production [6].

In contrast to the costimulatory signal derived from the binding of CD28 to B7-1 and B7-2, the engagement of CTLA-4 by these ligands provides a negative regulation of the immune response, as proved by the characterization of CTLA-4 deficient mice (CTLA-$4^{-/-}$). In fact, CTLA-$4^{-/-}$ mice showed lymphoproliferative disorders that led to neonatal death after 3-4 weeks of age, underscoring the central role of this receptor in induction of peripheral tolerance through a direct inhibition of CD28 signaling or by regulating the availability of cofactors necessary for TCR signaling [5]. Because of the lack of intrinsic enzymatic activity, CTLA-4 binds signaling molecules, such as protein phosphatase 2A (PPA-2) and Src homology phosphatase 2 (SHP-2), which mediate its effects [5]. CTLA-4 should be also involved in the regulation of $CD4^+CD25^+$ T regulatory cells (Tregs), which constitutively expressed this receptor on their surface [5]. Although still debated, part of the inhibitory function of CTLA-4 may result from its ability to enhance the generation of Tregs or, as an alternative, to modulate their functions. This hypothesis is supported by the evidence that mice with a Tregs-conditional deletion developed lymphoproliferative syndrome [7]. In addition, CTLA-4 blockade caused the abrogation of Treg functions *in vivo* [8].

The tumor microenvironment is often characterized by the presence of anergic T cells, due to the lack of positive costimulatory molecules, mainly B7-1 and B7-2, on the surface of cancer cells [9]. One strategy to revert this scenario is to force B7 expression on tumor cells, rendering them able to activate T-cell immune response.

Several studies showed that the induction of B7-1 on tumor cells was sufficient to stimulate the $CD8^+$ T cell-mediated rejection in several tumor models, as well as a memory response, but was insufficient to mediate rejection of poorly immunogenic tumors [4]. Several phase I clinical trials evaluated the efficacy of B7-1 transfected tumor cell vaccines, with or without IL-2, with encouraging preliminary results in patients affected by metastatic renal carcinoma and nonsmall cell lung cancer (Table 1) [10, 11]. In a phase II trial, 39 patients with metastatic renal carcinoma were vaccinated with B7-1-transfected autologous tumor cells in combination with systemic IL-2 [12]. The authors observed 3% pathologic complete response, 5% partial response, 64% stable disease, 28% disease progression, and a median survival of 21.8 months; similar results have been reported for IL-2 alone [12].

B7-1 was also included in vaccine along with specific antigens, such as carcinoembryonic antigen (CEA) (Table 1). Recently, in a phase II trial for metastatic colorectal cancer, Kaufman et al. used a nonreplicating canaripox virus vector (ALVAC) expressing CEA and B7-1 in combination with chemotherapy. The observed objective response rate of 40.4% was similar to that reported for chemotherapy alone [13]. Improvements on new vaccine strategies led to the generation of viral vectors expressing a triad of costimulatory molecules (TRICOMs), such as B7-1, intercellular adhesion molecule 1 (ICAM-1), and lymphocyte function-associated antigen 3 (LFA-3), along with CEA, mucin-cell-surface-associated 1 (MUC-1), and prostate-specific antigen (PSA) antigens, with promising results in preclinical studies and clinical trials, both in terms of efficacy and safety (Table 1) [14–18]. In this regard, a phase II randomized controlled trial of poxiviral-based PSA-targeted immunotherapy in patients with metastatic castration-resistant prostate cancer showed that the treatment was well tolerated and associated with 44% reduction in the death rate [19]. Recently, multi-target vaccine approaches were tested *in vitro*, resulting in enhanced antitumor immune response against hepatocellular carcinoma and glioma cell lines [20, 21].

Although several preclinical evidences proved the efficacy of B7-1-based therapeutic strategy in the induction of tumor antigen-specific T-cell response, meaningful clinical improvement has been limited. In addition to the existence of multiple mechanisms of immune resistance, a possible explanation for the lack of marked clinical benefits is that B7-1 and B7-2 also bind CTLA-4 with higher affinity than CD28; therefore, it is possible that the engagement of CTLA-4 by B7-1—expressing vaccine could limit its ability to activate T-cell immunity. This observation opens the door for a new therapeutic strategy: the specific blockade of CTLA-4 coinhibitory signal.

Several studies demonstrated that the blockade of CTLA-4 using anti-CTLA-4 antibodies was capable of inducing the

Table 1: Clinical trials of B7:CD28 costimulatory molecules.

Costimulatory molecule	Tumor model	Therapeutic strategy	Refs
B7-1	Metastatic renal carcinoma	Vaccination with B7-1-transfected autologous tumor cells in combination with systemic IL-2	[10–12]
	Nonsmall cell lung cancer	Vaccination with an adenocarcinoma cell line expressing B7-1 and human leukocyte antigen A1(HLA-A1) or A2	[11]
	Metastatic colorectal cancer	Vaccination with ALVAC vector expressing CEA and B7-1 in combination with chemotherapy	[13]
	CEA-expressing carcinoma, metastatic carcinoma, prostate cancer	Vaccination with TRICOM vector	[14–17]
CTLA-4	Metastatic melanoma, metastatic renal cancer, nonsmall cell lung cancer	MDX-010 Ab blockade of CTLA-4 alone or in combination with vaccine, IL-2, and chemotherapy	[32–44]
	Melanoma, metastatic colorectal cancer, advanced gastric cancer, and esophageal adenocarcinoma	CP-675, 206 Ab blockade of CTLA-4 alone or in combination with chemotherapy	[45–53]
PD-1	Hematological malignancies	CT-011 Ab blockade of PD-1	[95]
	Advanced solid cancer	MDX-1106 Ab blockade of PD-1	[97]
	Solid Tumors	MK 3475	[99]
	Cancers, multiple indications	MDX 1105-01	[99]

rejection of different types of established tumors in mice, such as colon carcinoma, fibrosarcoma, prostatic carcinoma, lymphoma, and renal carcinoma, along with a memory response [4]. Although effective as monotherapy in the treatment of small and immunogenic tumors, a combination of CTL-4 blockade with other immunotherapeutic strategies is needed to treat large and poorly immunogenic tumors. Combination of CTLA-4 blockade and irradiated tumor vaccine expressing GM-CSF results in tumor rejection and reduction of tumor growth in the B16 melanoma model. Similar results were reported for the poorly immunogenic SM1 mammary carcinoma line and a transgenic model of prostate carcinoma [4, 22]. Moreover, the combination of anti-CTLA-4 with DNA vaccine increased T-cell immune response against melanoma-associated antigens and induced B16 tumor rejection [23]. In the same tumor model, the CTLA-4 blockade combined with $CD25^+$ Treg depletion and vaccination was reported to be effective in inducing tyrosinase-related-protein-2-(TRP-2-) specific $CD8^+$ T cells and in rejecting larger tumor loads [24]. An increased antitumor immunity in B16 melanoma model was reported following CTLA-4 blockade with peptide vaccine and a synthetic oligodeoxynucleotide (ODN) as adjuvant [25]. The use of anti-CTLA-4 along with radiotherapy led to the improvement of survival rate in a mouse model of metastatic breast cancer, whereas CTLA-4 blockade in combination with chemotherapy provided clinical benefits in the murine myeloma model MOPC-315 [26, 27].

The encouraging results obtained in preclinical models led to the development of two fully humanized anti-CTLA-4 antibodies, MDX-010 (ipilimumab), and CP-675,206 (tremelimumab) (Table 1). Ipilimumab is an IgG1 with a plasma half-life of 12–14 days, and it has been approved by the FDA in March 2011 for the treatment of advanced melanoma (Bristol-Myers Squibb, Princeton, NJ, USA). Tremelimumab is an IgG2 with a plasma half-life of approximately 22 days (Pfizer, New York, NY, USA). Both agents are able to recognize human CTLA-4 and block the interaction of CTLA-4 with CD80 or CD86, but their exact mechanism of action is not fully understood. Recently, some authors reported that tremelimumab induces a significant increase in $CD8^+$ cells intratumoral infiltration and that the immune response mediated by this agent is due to a direct activation of effector T cells rather than a depletion of Tregs [28, 29]. Preliminary data about an increase in antigen-specific effector T cells following ipilimumab treatment in combination with vaccine in three melanoma patients are also published [30].

Ipilimumab and tremelimumab were tested in ovarian, breast, prostate, colon carcinoma, and, mainly, in melanoma and renal cell cancer clinical trials [31]. Several early phase II studies evaluated ipilimumab in metastatic melanoma, reporting an objective response rate ranging from 6% to 21% and a disease-control rate of about 30% [32–34]. Recently, a multicenter single arm phase II study evaluated the efficacy and the safety of ipilimumab monotherapy in patients with pretreated advanced melanoma. Patients ($n = 155$) were treated with ipilimumab at 10 mg/kg and the authors showed that one- and 2-year survival rates were 47.2% and 32.8%, respectively, with a median overall survival of 10.2 months [35]. Moreover, a randomized, double-blind, placebo-controlled, phase II trial of ipilimumab at 10 mg/kg, with or without budesonide, in 115 patients with unresectable stage III or IV melanoma, showed that 2-year survival rate was approximately 40% in each arm [36]. In another randomized, double-blind, multicenter, phase II, dose-ranging study, 217 melanoma patients were randomly assigned to receive ipilimumab at 10 mg/kg ($n = 73$),

3 mg/kg ($n = 72$) or 0.3 mg/kg ($n = 72$). The authors observed a dose-dependent antitumor effect of ipilimumab, with the best overall response of 11.1% in patients treated with 10 mg/kg [37]. Ipilimumab was also tested in combination with other therapeutic agents such as IL-2, vaccine or chemotherapy, such as dacarbazine [38–42]. In particular, interesting results came from the phase III trial by Hodi et al.; in this study, melanoma patients were randomized to receive ipilimumab 3 mg/kg, with or without a gp100 peptide vaccine, or the vaccine alone. The primary endpoint of the study was the overall survival. The median overall survival was approximately 10.0 months among patients receiving ipilimumab (with or without gp100 vaccine), as compared with 6.4 months among patients receiving gp100 alone [41]. In another phase III study, the administration of ipilimumab (at a dose of 10 mg/kg) in combination with dacarbazine was associated with a significant improvement in overall survival among patients with previously untreated metastatic melanoma [42]. Ipilimumab was also tested in other types of malignancies. In a phase II study on 61 patients affected by metastatic renal cell carcinoma, ipilimumab was administrated at a dose of 1 mg/kg or 3 mg/kg; five of 40 patients treated with 3 mg/kg had a partial response [43]. A recent phase II study compared the addition of ipilimumab to carboplatin and paclitaxel chemotherapy in nonsmall cell lung cancer patients. Ipilimumab was administered either concurrently or in a phased schedule after receiving the first two cycles of chemotherapy. Patients treated with ipilimumab plus chemotherapy, in concurrent and sequential regimens, showed an improved overall survival compared with patients receiving chemotherapy alone [44]. Tremelimumab was first evaluated in the treatment of metastatic melanoma. A phase I clinical trials evaluating the maximum-tolerated dose of tremelimumab showed antitumor activity of this drug in melanoma patients [45]. Other phase I/II studies reported SD occurring in about 30% of melanoma patients treated with tremelimumab and an objective antitumor response in 10% of patients [46–48]. A phase II study evaluated the antitumor activity of 15 mg/kg tremelimumab in 246 patients affected by melanoma, reporting an objective response rate of 6.6%, with duration of response ranged from 8.9 to 29.8 months [49]. A phase III trial of tremelimumab in combination with DTIC or temozolomide was recently withdrawn, because of the lack of a survival advantage in the tremelimumab group [50]. Tremelimumab was also tested in cancers others than melanoma, both as monotherapy and in combination therapy, such as metastatic renal cell carcinoma, metastatic colorectal cancer, and advanced gastric and esophageal adenocarcinoma, but not significant clinical improvements were reported [51–53]. The treatment with both ipilimumab and tremelimumab is associated with inflammatory adverse events like rash, diarrhea, colitis, and hypophysitis; this side effect profile could be linked to the potentiation of Th17 cell differentiation following CTLA-4 blockade [54].

2.2. ICOS-L:ICOS. The inducible costimulator (ICOS, CD278) is a costimulatory receptor that is weakly expressed on naïve T cells and quickly upregulated in activated $CD4^+$ and $CD8^+$ T cells. A constitutive expression of ICOS by $CD25^+CD4^+Foxp3^+$ Tregs has also been reported [55]. The cognate ligand for ICOS is ICOS-L (B7h, B7RP-1, CD275), which is expressed by professional APCs and by peripheral epithelial and endothelial cells following TNF-α stimulation [56]. The ICOS:ICOS-L pathway provide a key costimulatory signal for T-cell proliferation and, mainly, for T-cell survival [57]. Moreover, ICOS regulates development and response of T follicular helper (Tfh), Th1, Th2, Th17 cells and plays roles in the maintenance of memory effector T cells and Tregs homeostasis [57]. The observation that immune defects in CD28 knockout mice can be reverted by crossing them with *sanroque* mice, which express ICOS constitutively, suggested that the two receptors activated similar intracellular pathways [58]. In fact, ICOS is able to trigger the PI3K/Akt pathway greater than CD28 and to activate the downstream MAPKs cascade [59]. Due to its role in sustaining T-cell activation and effector functions, targeting ICOS:ICOS-L could represent a plausible approach to enhance antitumor immunity. The ICOS-L costimulation, through its expression on tumor cells, was capable of inducing cancer regression in Sa1/N fibrosarcoma and J558 plasmacytoma models [60, 61]. Systemic treatment with murine ICOSL-IgG fusion protein was effective in promoting INF-γ-dependent antitumor immunity in MethA fibrosarcoma and B16F1 melanoma tumor models [62]. Recent data showed an increase of $ICOS^{hi}$ $CD4^+$ effector T cells percentage after CTLA-4 blockade in several cancer models. In addition, upon CTLA-4 blockade, this cell population produced greater levels of INF-γ than $ICOS^{lo}$ $CD4^+$ T cells, suggesting that ICOS could be used as a marker for $CD4^+$ effector T-cell response [63–66]. *A downregulation of ICOS was shown in colon cancer patients and* the expression of ICOS in tumors was associated with a greater survival of melanoma patients [67, 68]. A recent study investigated the role of ICOS in the Tregs in melanoma, demonstrating the selective expression of ICOS on a "hyperactivated" Treg population that strongly inhibits T-cell response through IL-10-mediated APCs suppression [69]. Moreover, ICOS-L was expressed by both cultured and freshly isolated melanoma cells from stage IV melanoma patients and could provide costimulation through ICOS for the activation and expansion of Tregs in the tumor microenvironment, as another mechanism of escape from immune surveillance [70]. Thus, targeting costimulatory and coinhibitory molecules on Tregs might be a promising approach for modulating peripheral tolerance in cancer patients.

2.3. PD-L1/PD-L2:PD-1. Programmed cell death 1 (PD-1) is a negative costimulatory receptor belonging to the B7/CD28 family. PD-1 expression is induced on activated T cells, B cells, monocytes, DCs, and, at low levels, on natural killer T cells (NKT) [71]. Negative costimulatory signals mediated via PD-1 and CTLA-4 are not redundant; in fact PD-1 mainly acts in regulating inflammatory responses in peripheral tissues, whereas CTLA-4 modulates T-cell priming in lymphoid organs. In addition, in contrast to CTLA-4, PD-1 is able to block TCR- and CD28-mediated activation through the recruitment of inhibitory phosphatases, such as SHP-2, which inhibits the induction of PI3K activity [71].

PD-1 has two known ligands belonging to the B7 family: PD-L1 (B7-H1) and PD-L2 (B7-DC). To date, one of the major differences between these ligands concerns their expression pattern. PD-L1 mRNA is broadly expressed in multiple peripheral tissues such as heart, placenta, muscle, fetal liver, spleen, lymph nodes, and thymus; PD-L1 protein has been found in activated T cells, B cells, monocytes, DCs, in endothelial cells and myocardium and can be upregulated following exposure to type I or type II interferon, providing a negative feedback mechanism to dampen immune response [71]. On the contrary, PD-L2 expression is largely restricted to activated macrophages and DCs, but only PD-L2 mRNA can also be observed in the human heart, placenta, lung, and liver [72]. The function of PD-L1 and PD-L2 has been debated, owing to conflicting results about the costimulatory signal provided by the ligands. Opposite results have been obtained using PD-L1 fusion protein and mAbs, but, to date, the accepted opinion is that PD-1/PD-L1 interaction generally produces a negative costimulatory signal [69]. The same controversial results has been reported about PD-L2, with one group demonstrating a negative costimulatory role for this ligand, and another group reporting that PD-L2 was able to stimulate T cells [72]. *In vivo* data from PD-L2 deficient mice supported a stimulatory function of PD-L2. Anyway, the disagreeing results concerning PD-L1 and PD-L2 roles may be explained by the existence of another receptor able to bind these ligands that have positive costimulatory functions, like CD28 [73].

PD-L1 aberrant expression has been reported in many human cancers, such as glioblastoma, melanoma, and cancers of the head and neck, lung, ovary, colon, stomach, kidney, and breast [74, 75]. Moreover, in several follow-up studies, the expression of PD-L1 correlates with a poor prognosis of patients [76–82]. Based on these experimental evidences, PD-L1 blockade has been proposed for cancer immunotherapy. Two independent studies have shown that forced expression of PD-L1 in the murine myeloma cell line P815 render them more resistant to *in vitro* cytolysis and less susceptible to rejection than control when inoculated in mice [83, 84]. In addition, the treatment with anti-PD-L1 mAbs was capable of inhibiting the growth of P815-PD-L1 *in vivo* [84]. PD-1 blockade was able to restore the antitumor immunity to accelerate tumor eradication in murine squamous cancer cell line SCCVII, in P815 cell line and to block both CT26 colon carcinoma metastasis to the lung and B16 melanoma metastasis to the liver [85–87]. *In vivo* studies in these tumor models examined the combination of anti-PD-1 mAb with GM-CSF-secreting tumor cell vaccine and reported that the administration of anti PD-1 mAb enhanced the efficacy of vaccine increasing number and activity of tumor-specific $CD8^+$ T cells [88]. A recent study reported that the combination of PD-1 blockade and NKT cell activation results in increased antitumor responses in a melanoma model [89]. A reduced number of Tregs at the tumor site was observed after the treatment with anti-PD-1 mAb and the Toll-like receptor agonist CpG, suggesting that it could be another mechanism by which PD-1 blockade exerts antitumor effects [90]. The expression of PD-L1 on DCs is also able to block antitumor T-cell response; myeloid DCs expressing PD-L1 isolated from ovarian cancer poorly stimulated T cells and PD-L1 blockade could revert this scenario [91]. In addition, the anti-PD-L1 therapy was observed to revitalize "exhausted" antiviral $CD8^+$ T cells in animals with chronic viral infections [92]. The promising results in preclinical models have led to the development of two humanized antibodies against PD-1 receptor that block its interaction with PD-L1:CT-011 and MDX-1106 (Table 1). A preclinical study evaluated the combination of CT-011 with low dose of cyclophosphamide and with a tumor vaccine; the authors reported the complete regression of established tumors in most of the animals treated [93]. Benson et al. reported that CT-011 enhances NK-cell migration toward malignant plasma cells in multiple myeloma [94]. To date, only phase I clinical trials have been conducted to evaluate both efficacy and safety of these two agents. In a phase I trial enrolling 17 patients with advanced hematological malignancies, the treatment with CT-011 at doses ranging from 0.2 to 6 mg/kg led to clinical improvement in 33% of patients [95]. Recently, the administration of CT-011 in combination with autologous dendritic cell/myeloma fusion-vaccine was demonstrated to stimulate T-cell responses after vaccine administration [96]. The efficacy of MX-1106 was evaluated in a phase I study enrolling 39 patients with advanced solid cancers (melanoma, colorectal cancer, castrate-resistant prostate cancer, nonsmall-cell lung cancer, and renal cell carcinoma) obtaining very promising results; phase II and III trials are under evaluation [97]. In particular, it will be interesting to evaluate the combination of anti-PD1 with other agents, such as anti-CTLA-4 mAb, vaccines, and chemotherapy. Several phase II clinical trials are testing the safety and the efficacy of these two PD-1 antibodies in several types of cancer [98]. MK-3475 and MDX-1105-01 are other two antibodies against PD-1 and PD-L1, respectively, which are currently being investigated in phase I clinical trials (Table 1) [99].

2.4. HVEM:BTLA/CD160. The B- and T-lymphocyte attenuator (BTLA) is another member of the B7/CD28 family acting as a negative costimulatory receptor [100]. The constitutive expression of BTLA has been reported, at low levels, on naïve B and T cells, Tfh, macrophages, DCs, NKT cells, and natural killer cells (NK), but unlike CTLA-4 and PD-1, BTLA is not expressed on Tregs [100]. BTLA expression is upregulated following T-cell activation [101]. Moreover, like PD-1, BTLA seems to have a role in inducing $CD8^+$ T-cell exhaustion during chronic viral response. The herpes virus entry mediator (HVEM), a member of TNFR superfamily, has been identified as BTLA ligand. HVEM expression is high in naïve T and B cells, but it decreases during T-cell activation. HVEM is also expressed on DCs, Tregs, monocytes, NK cells, and neutrophils, and in nonhematopoietic cells, such as parenchymal cells [100].

In addition to BTLA, HVEM binds also CD160, another member of the B7/CD28 family. CD160 is highly expressed on $CD56^{dim}CD16$ NK cells, NKT cells, $\gamma\delta$ T cells, $CD8^+CD28^-$ T cells, a small subset of $CD4^+$ cells and intestinal intraepithelial T cells (IEL) [100].

HVEM also interacts with LIGHT (described below in the text) and lymphotoxin alpha (LTα) of TNFR family, being the unique example of a direct interaction between the two families [102]. Therefore, HVEM is considered a molecular switch, because of its ability to regulate the immune response depending on which cognate ligand binds. In contrast to LIGHT and LTα engagement, which generally delivers positive costimulatory signals, HVEM engagement of BTLA and CD160 provides negative costimulatory signals to T cells [100, 102]. The role of BTLA as a negative costimulatory receptor has been shown by the phenotype of BTLA deficient mice, which were more susceptible to develop autoimmune disorders, and by the *in vitro* observation that anti-BTLA agonists drive negative signals to T cells [102]. The engagement of BTLA results in the inhibition of CD3/CD28 T-cell activation. BTLA signals through the recruitment of SHP-2 phosphatase, but the downstream target of SHP-2 is unclear. Anyway, recent studies showed that triggering BTLA signaling in B cells resulted in blocking B-cell proliferation through the inhibition of phosphorylation of some transcription factors like NF-κB [103].

The similarity between BTLA and PD-1 signaling could justify a possible use of BTLA blockade to enhance antitumor immunity. The expression of BTLA was found in chronic lymphocytic leukemia/small lymphocytic lymphoma [104]. Moreover, soluble BTLA seems to enhance antitumor efficacy of the HSP70 vaccine in murine TC-1 cervical cancer mice [105]. Recently, a study by Derré et al. demonstrated the potentiality of targeting BTLA for cancer immunotherapy, reporting that BTLA is expressed on tumor antigen-specific $CD8^+$ T cells from melanoma patients and that this molecule inhibits their fully functionality; following the vaccination with CpG adjuvants, the authors observed a downregulation of BTLA, with a partial recovery of $CD8^+$ T cells functionality [106]. BTLA-HVEM blockade showed antitumor effects in murine TC-1 cervical cancer model *in vivo*, resulting in downregulation of IL-10 and TGF-beta and in activation of dendritic cells in IL-12- and B7-1-dependent manner. Anyway, BTLA-HVEM blockade alone was not effective in eradicating the tumor, whereas the combination with HSP70 vaccine improved antitumor immunity by increasing IL-2 and INF-γ production and decreasing IL-10, TGF-beta, and Foxp3 transcription levels in the tumor microenvironment [107]. The evidences supporting a negative costimulatory function for CD160 come from *in vitro* studies, because CD160 deficient mice have not been generated. CD160 agonists strongly inhibited $CD4^+$ T-cell proliferation and cytokines production and reduced INF-γ secretion by NK cell line [108]. Recently, Cai et al. reported a strong inhibition of CD3/CD28-induced T-cell activation after the use of CD160 agonists, but the downstream intracellular pathways involved are not known [109]. Indeed, Liu et al. reported that CD160 is expressed in B-cell chronic lymphocytic leukemia, in which its engagement mediates survival and growth signals. In fact, CD160 activation was associated with upregulation of antiapoptotic genes Bcl-2, Bcl-xL and Mcl-1 and, consequently, with reduced mitochondrial membrane potential collapse and cytochrome c release. CD160 engagement also induced cell cycle progression and proliferation [110]. A recent study examined the expression of CD160 in 811 cases of B-cell lymphoproliferative disorders (B-LPD). The authors showed that CD160 was expressed in 98% of chronic lymphocytic leukemia (CLL) cases, 100% of hairy cell leukemia (HCL) cases, 15% of mantle cell lymphoma (MCL) in the leukemic phase, and 16% of other B-LPD cases, whereas it was absent in the normal B-cell lineage [111]. Recently, Chabot et al. suggested a role for CD160 in tumor neoangiogenesis. CD160 was expressed on newly formed blood vessels in human colon carcinoma and mouse B16 melanoma, but not in the healthy vessels. Treatment with anti-CD160 monoclonal antibody CL1-R2 in combination with cyclophosphamide chemotherapy resulted in the regression of tumor vessels in B16 melanoma-bearing mice [112]. Further studies are needed to clarify this pathway so as to design potential CD160/BTLA-based antitumor therapeutic strategies.

2.5. B7-H3 and B7-H4. B7-H3 and B7-H4 (B7x, B7S1) are two of the newer members of the B7-family. B7-H3 expression has been found to be inducible on T cells, NK cells and APCs [113]. B7-H3 is also broadly expressed on osteoblasts, fibroblasts, and epithelial cells, as well as in liver, lung, bladder, testis, prostate, breast, placenta, and lymphoid organs [113]. To date, only one potential receptor of B7-H3 on activated T cells named TLT-2 has been identified [113]. There are conflicting data about the functions mediated by B7-H3, as both stimulatory and inhibitory properties have been reported. Initial studies described B7-H3 as a positive costimulatory molecule which enhanced the proliferation of both $CD4^+$ and $CD8^+$ T cells, the induction of cytotoxic T lymphocytes (CTLs) and the production of INF-γ. By contrast, other studies suggested an opposite role for B7-H3, showing that it is able to inhibit T-cell activation and cytokines production, like IL-2 [114]. Moreover, B7-H3 blockade with antagonistic mAbs enhanced T-cell proliferation *in vitro* and worsened the experimental autoimmune encephalomyelitis (EAE) *in vivo*, an autoimmune disorder observed also in B7-H3 deficient mice [114]. Because of these controversial results, the possible existence of additional receptors for B7-H3 has been taken into consideration.

Recently, several works reported B7-H3 expression in different human cancers, as reviewed by Loos et al. The double stimulatory and inhibitory nature of B7-H3 signaling also appeared in murine cancer models and in human cancers. The authors showed that B7-H3 expression is associated both to favorable and adverse clinicopathologic features [113]. Due to its immunomodulatory ability, B7-H3 blockade could be a potential anticancer immunotherapy, but the controversial findings about its role remain to be elucidated.

B7-H4 mRNA is broadly expressed in the peripheral tissues, whereas protein expression is restricted to activated B cells, T cells, and monocytes [115]. To date, the cognate receptor of B7-H4 on activated T cells remained unclear, although BTLA has been reported as a possible receptor. Unlike B7-H3, which shows opposite functions, B7-H4 mediates a negative costimulatory signal [116]. B7-H4 strongly inhibited T-cell proliferation and IL-2 secretion, and its blockade with antagonistic mAbs resulted in *in vitro*

enhanced T-cell response and *in vivo* exacerbation of EAE [115, 116].

Several studies reported the expression of B7-H4 in many human cancers, such as nonsmall cell lung cancer, ovarian cancer, prostate cancer, breast cancer, and renal cancer [75]. Recent studies indicate that B7-H4 could be a potential diagnostic/prognostic marker and/or therapeutic target for several cancers. In fact, B7-H4 expression was found to correlate with stage, pathological types, and biological behavior of many tumors, as shown by retrospective analyses on 13 types of human cancers. Moreover, the expression of B7-H4 reverse correlated with the survival of patients in most cancer analyzed [117]. A recent study by Quandt et al. demonstrated that the overexpression of B7-H4 in melanoma cells impaired the antitumor immune response by decreasing IFN-γ, TNF-α, and IL-2 production [118]. In ovarian cancer, the inhibitory role of B7-H4 might be due to B7-H4 expression on tumor-associated macrophages (TAMs). Kryczek et al. showed that B7-H4-expressing TAMs could block activation of T cells in the setting of ovarian cancer. B7-H4 expression in TAMs was upregulated by IL-6 and IL-10 present in the tumor microenvironment, whereas its expression was inhibited by GM-CSF and IL-4. It has been shown that Treg cells induced APCs to produces IL-10 and IL-6, providing a new mechanism by which Tregs exert their suppressive action. B7-H4 blockade by antisense oligonucleotides restored T-cell antitumor immune response and led to tumor regression *in vivo* [119].

Recently, Qian et al. reported the development of a monoclonal antibody to B7-H4 and preliminary data showed that it could effectively inhibit the activity of B7-H4, promoting the growth of T cells and the secretion of IL-2, IL-4, IL-10, and IFN-γ [120]. Based on these preliminary data, the blockade of B7-H4 could be an attractive opportunity to enhance antitumor immunity in a subset of human cancers.

3. The TNF:TNFR Family

3.1. CD40L:CD40. CD40 receptor and its ligand CD40L (CD154) were the first members belonging to the TNF:TNFR family to be identified [121]. CD40 expression has been originally found on B cells [122], but it is also expressed on DCs, monocytes, platelets, macrophages as well as myofibroblasts, fibroblasts, epithelial, and endothelial cells [121]. CD40L is expressed on activated T and B cells, by platelets, monocytes, NK cells, mast cells and basophils, where CD40L is induced following proinflammatory stimuli [121]. The engagement of CD40 by CD40L on APCs has been shown to promote cytokines production and upregulation of costimulatory molecules, crucial events for T-cell activation, and differentiation [123]. CD40L:CD40 signaling in B cells is also important for the generation of long-lived plasma cells and memory B cells, as well as for their survival. CD40 intracellular signaling is mediated by the recruitment of TNFR-associated factors (TRAFs), which in turn activate different pathways, such as the canonical and noncanonical NF-κB pathway, MAPKs, PI3K and the phospholipase Cγ pathway [121].

CD40/CD40L pathway is crucial for the development of antitumor immunity. CD40L blockade resulted in lacking of protective immune response following administration of a GM-CSF-expressing B16 melanoma cells vaccine. In addition, low expression of CD40L was sufficient to induce a long-lasting antitumor immune response via CTLs in a small number of cancers [4, 121]. The combination of CD40L expression with other immunomodulators (IL-2, GM-CSF, and INF-γ) has been found to promote antitumor immunity in several cancer models. Gene therapy approach with the use of recombinant adenovirus encoding CD40L was also effective in colorectal, lung, and melanoma cancer models. An early study reported that the use of CD40 agonistic antibodies triggered CTL-4 responses in a lymphoma system, with the consequent tumor eradication [4, 124]. Recently, the use of activators of both adaptive and innate immunity, such as CD40 agonists and Toll-like receptor (TLR) agonists, induced antitumor-specific immunity in many tumor models [125]. Currently, the combination of CD40 tumor therapy with other approaches, such as cancer vaccines, chemotherapy, radiation, CTLA blockade, TLR agonists, and cytokines, is becoming overriding [125–127].

Another aspect to take into consideration is the direct effect of CD40:CD40L pathway on tumor cells. Elgueta et al. reviewed that CD40 is broadly expressed in several tumors, such as melanoma, prostate cancer, lung cancer, as well as carcinoma of nasopharynx, bladder, cervix and ovary, Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, and acute myeloid leukemia. The engagement of CD40 on tumor cells can provide growth arrest and apoptosis of malignant cells, dependently on type of malignancies and the microenvironment [121].

To date, three humanized CD40 agonistic antibodies have been developed: CP-870,893, SGN-40, and HCD 122 (Table 2). CP-870,893 is a fully human, IgG2 antibody that selectively binds to CD40. It enhances the expression of MHC class II, CD54, CD86 and CD23 on human B cells *in vitro*. CP-870,893 also enhances dendritic cell activity as demonstrated by secretion of IL-12, IL-23, and IL-8, by the upregulation of CD86 and CD83, and by the capacity to prime T cells to secrete IFN-γ [128, 129]. Results from a phase I study showed that administration of CP-870,893 was associated with early signs of clinical efficacy, especially in patients with melanoma [130]. The same authors reported that weekly infusions of this agonist CD40 antibody were associated with little clinical activity in advanced cancer patients [131]. SGN-40 (Dacetuzumab) is a humanized anti-CD40 monoclonal antibody with multiple mechanisms of action. In non-Hodgkin lymphoma, Dacetuzumab activates two distinct proapoptotic signaling pathways; on the one hand, it constitutively activates the NF-κB and MAPK signaling pathways producing the sustained downregulation of the oncoprotein B-cell lymphoma 6, which loss results in c-Myc downregulation and activation of early B-cell maturation, concomitant with reduced proliferation and cell death. On the other hand, dacetuzumab induces the expression of the proapoptotic p53 family member TAp63α and downstream proteins associated with the intrinsic and extrinsic apoptotic machinery [132]. *In vitro*, dacetuzumab exhibited

Table 2: Clinical trials of TNF:TNFR costimulatory molecules.

Costimulatory molecule	Tumor model	Therapeutic strategy	Refs
CD40	Melanoma, Advanced cancers	CP-870,893 agonistic Ab anti-CD40	[130, 131]
	Myeloma, B-cell lymphoma, diffuse large cell lymphoma	SGN-40 agonistic Ab anti-CD40	[134–136]
	Multiple myeloma, B-cell chronic lymphocytic leukemia	HCD 122 agonistic Ab anti-CD40	[121]
4-1BB	Solid tumors	BMS-663513 agonistic Ab anti-4-1BB	[148]
OX40	Metastatic prostate cancer	Agonistic Ab anti-OX40	[99]
GITR	Melanoma	TRX518	[99]
CD30	CD30-positive lymphoma	Brentuximab vedotin Ab-drug conjugates anti-CD30	[179–182]

antitumor activity against several B-cell lymphoma and multiple myeloma (MM) cell lines, and induced direct apoptosis as well as the engagement of effective antibody-dependent cell-mediated cytotoxicity (ADCC) [133]. Early clinical trials have evaluated the pharmacokinetics, safety and efficacy of dacetuzumab in patients with relapsed/refractory B-cell lymphomas, MM and chronic lymphocytic leukemia [134–136]. Dacetuzumab resulted in modest antitumor activity in B-cell lymphomas and, to a lesser extent, in MM. In chronic lymphocytic leukemia dacetuzumab showed modest activity as monotherapy, while better results were obtained by using combination therapy with lenalidomide [137]. Dacetuzumab is currently in multiple phase II trials for the treatment of myeloma and diffuse large cell lymphoma. HCD122 is a human IgG1 monoclonal antibody. In B-cell chronic lymphocytic leukemia, HCD122 exerts antitumor activity by killing leukemia cells through ADCC and inhibiting CD40L-induced survival and proliferation of tumor cells [138]. HCD 122 is in a phase I trial for the treatment of multiple myeloma and B-cell chronic lymphocytic leukemia [121].

3.2. 4-1BBL:4-1BB. 4-1BB is an inducible costimulatory receptor expressed on activated $CD4^+$ and $CD8^+$ T cell, NKT, NK cells, DCs, macrophages, eosinophils, neutrophils, and mast cells, as well as Tregs [139, 140]. In the most cases, 4-1BB is induced on the cellular surface following activation, except for APCs and Tregs, where its expression is constitutive [139]. The ligand of 4-1BB is 4-1BBL, which is expressed on activated professional APCs [139]. 4-1BB:4-1BBL pathway seems to amplify the existing costimulatory signals, even if the engagement of 4-1BB in the presence of a strong TCR signaling can induce IL-2 production in a CD28-independent manner [141]. Following stimulation with its ligand, 4-1BB provides costimulatory signals to both $CD4^+$ and $CD8^+$ T cells, with a greater effect on the expansion of $CD8^+$ due to the upregulation of antiapoptotic genes, such as *bcl-*X_L and *bfl-1*. 4-1BB signals are mediated by the activation of NF-κB, c-Jun and p38 downstream pathways [142]. 4-1BB has also a role in activation of DCs, inducing IL-6 and IL-12 production and upregulating B7 costimulatory molecules [142]. 4-1BB plays roles in activating non-T-cells other than DCs, such as monocytes, B cells, mast cells, NK cells, and neutrophils and its engagement is associated with cellular proliferation, cytokine induction, bactericidal activity and sustenance of T-cell effector functions [139].

Targeting of 4-1BB:4-1BBL pathway in cancer reveals itself as a promising approach. The adoptive transfer of *ex vivo* 4-1BB- and CD28-costimulated T cells induced antitumor immune response in some preclinical studies [143, 144]. Nevertheless, this approach seems not to be a practicable way, because of the limits of this application in humans, such as the small number of *ex vivo* generated T cells and the risk of transformation of T cells during *in vitro* culture [142]. 4-1BB agonistic antibodies as antitumor therapy were broadly tested in several animal models with encouraging results. Melero et al. reported that the intraperitoneal injection of an antimurine 4-1BB mAb resulted in the eradication of established P815 mastocytoma and Ag104A sarcoma in mice [145]. Driessens et al. reviewed of subsequent studies that demonstrated the efficacy of anti-4-1BB- or 4-1BBL-expressing tumor cells vaccines in inducing specific antitumor T-cell response, suppression of tumor growth and regression of preestablished tumors in different animal models [4]. Therapeutic effects of agonistic anti-4-1BB mAb are due to enhanced natural killer (NK) and $CD8^+$ T-cell activation and IFN-γ production [140]. The current direction toward which 4-1BB-directed anticancer immunotherapy is moving is the use of anti-4-1BB mAbs in combination with other therapeutic approaches, such as antitumor necrosis factor-related apoptosis inducing ligand (TRAIL), CD40 mAbs, intratumoral delivery of IL-12 gene, DC vaccines, CTLA-4 blockade, anti-CD4 therapy, chemotherapy, and radiotherapy [4, 146, 147]. To date, one agonistic anti-4-1BB-humanized mAb BMS-663513 has been developed and the functional effects were demonstrated on human and monkey T cells and peripheral blood mononuclear cells (PBMCs), where IFN-production was enhanced compared to controls (Table 2) [148]. BMS-663513 is under evaluation in several phase I and II trials in patients with solid tumors, showing clinical activity. A phase II randomized study in melanoma patients with stage IV disease was stopped due to the occurrence of hepatitis [98, 148, 149].

3.3. OX-40:OX-40L. OX-40 is an inducible costimulatory receptor expressed on activated $CD4^+$ and $CD8^+$ T cell, but also on activated Tregs, NKT cells, NK cells, and neutrophils

[150]. OX-40L expression is induced on professional APCs, as well as on T cells, with the aim of amplifying T-cell responsiveness during T-cell/T-cell interactions [150]. In addition to APCs, other cell types can induce OX-40L expression, such as Langerhans cells, mast cells, NK cells, endothelial cells, and smooth muscle cells [150]. Based on experimental evidences from OX-40 deficient mice, it has been reported that this receptor promotes effector T-cell proliferation and survival, cytokines production, as well as the generation, and the maintenance of memory T cells [150]. Moreover, OX-40 inhibits Treg functions and counteracts the generation of inducible Tregs [150]. OX-40 seems to act as a late positive costimulatory receptor, which goes on after CD28 signal, in a sequential manner [151]. The pro-survival activity of OX-40 is in part due to its ability to upregulate antiapoptotic genes of the Bcl-2 family. In fact, OX-40 engagement by OX-40L activates both PI3K/Akt and NF-κB downstream pathways [152].

OX-40 represents a promising candidate for cancer immunotherapy. As reviewed by Croft et al., different approaches have been evaluated, such as agonistic OX-40 mAbs or OX-40L-Ig fusion protein, tumor cells and DCs transfection with OX40L, and agonist RNA aptamer-binding OX-40. Treatment with agonistic OX-40 mAbs or OX-40L-Ig fusion protein resulted in enhanced antitumor immunity in several cancer models, such as sarcoma, melanoma, colon carcinoma, and glioma [150]. Several studies also reported encouraging results following the use of agonistic OX-40 mAbs in combination with IL-12, anti-4-1BB, GM-CSF, DC vaccine, IL-12, and CD80 costimulation and chemotherapy [153–156]. Combination therapy with agonistic OX40 mAbs and cyclophosphamide induces a profound Tregs depletion in concomitance with an increased infiltration of effector $CD8^+$ T cells in B16 melanoma model [157].

A phase I/II trial is ongoing to evaluate the safety and the efficacy of a murine anti-human OX40 in combination with cyclophosphamide and radiation in patients with progressive metastatic prostate cancer (Table 2). To avoid immune response to the murine mAb, a humanized OX-40 agonist has been developed by Agonox, a spinoff biotech company, and it will be tested in future clinical trials [99].

3.4. Light:HVEM. LIGHT, along with LTα, was identified as a ligand of the aforementioned HVEM receptor and it is a member of TNF family [101]. LIGHT expression has been reported on activated T cells, on immature DCs, on monocytes and NK cells. LIGTH is not expressed on B cells, but it can be induced following activation [101]. Experimental evidences suggested that the interaction HVEM/LIGHT results in a positive costimulatory signaling, inducing T-cell proliferation and cytokines production [102]. In fact, the constitutive expression of LIGHT in T cells of transgenic mice leads to accumulation and activation of DCs and expansion of activated effector and memory T cells [158]. Moreover, the manifestation of lymphoproliferative disorders and autoimmune disease was also observed [159]. LIGHT deficient mice have been generated and showed defects in $CD8^+$ T-cell activation and in thymic selection. LIGHT is also a critical ligand for activating NK cells to produce IFN-γ [101]. The intracellular signaling of HVEM following LIGHT binding is mediated by TRAF proteins, which in turn activate NF-κB and c-Jun/AP-1 pathways, leading to the transcription of prosurvival and proproliferative genes, as well as genes regulating cytokines secretion [102]. LIGHT can also engage the lymphotoxin-β-receptor (LTβR) on DCs and provide a crucial signaling resulting in DCs expansion, activation and IL-2 production [102]. Thus, LIGHT can modulate the immune response both directly by signaling via HVEM on T cells and indirectly by activating DCs through LTβR. Due to its role as immunomodulator, LIGHT could be a suitable target for cancer immunotherapy. The overexpression of LIGHT in P815 myeloma cell line induces regression of established tumors in a CD28-independent manner [160]; similar results were obtained upon LIGHT overexpression in Ag104 sarcoma cell line; in fact, this forced expression caused rejection of tumor through NK-cell activation, which, in turn, triggered tumor-specific $CD8^+$ T-cell proliferation at the tumor site [161]. In addition, the injection of LIGHT-expressing adenoviral vector into primary 4T1 mammary carcinoma has been found to promote T-cell recruitment, immune surveillance of the tumor, and elimination of metastasis [162]. A recent study showed that the LIGHT/HVEM costimulation through both LIGTH-transfected cells and HVEM agonistic mAb-induced apoptosis in fresh B-chronic lymphocytic leukemia cells along with an increased production of IL-8 [163]. Another therapeutic approach targeting LIGHT/HVEM signaling was reported by Park et al., which developed P815 tumor cell expressing a single-chain variable fragment (scFv) of an anti-HVEM agonistic monoclonal antibody on their surface. These authors showed that tumor cells expressing anti-HVEM scFv spontaneously regress in a $CD4^+$ and $CD8^+$ T cell-dependent manner when inoculated in mice and stimulated tumor-specific long-term T-cell memory. Moreover, the combination of anti-HVEM scFv-expressing tumor vaccines and 4-1BB costimulation caused the regression of established tumors *in vivo* [164]. Further studies are needed to clarify the true potential of targeting this pathway in cancer immunotherapy.

3.5. CD70:CD27. CD27 is another costimulatory receptor belonging to the TNF family, and it is expressed on naïve T and B cells and on NK cells [151]. CD70 has been identified as CD27 ligand and its expression is restricted to APCs [151]. The engagement of CD27 by CD70 promotes a positive costimulatory signaling, resulting in T-cell proliferation and survival, maybe in concert with CD28 [151]. Recently, a critical role for CD70 in priming $CD8^+$ T cells has been demonstrated [165]. The stimulatory role of this pathway is confirmed by the observation that CD70 and CD27 transgenic mice developed autoimmune diseases [166]. Like other members of the TNFR family, CD27 signaling is mediated by the recruitment of TRAF proteins [167]. Targeting CD27 could represent an attractive strategy in the field of cancer immunotherapy. Driessens et al. reviewed about early studies reporting that the overexpression of CD70 promoted cancer elimination through the activation of T cells and NK cells [4]. In addition, the potential of costimulatory ligand CD70 to boost DC-based vaccine capacity to evoke effective

$CD8^+$ T-cell immunity has been explored [168]. Glouchkova et al. suggested that the modulation of the CD70/CD27 pathway might represent a novel therapeutic approach for enhancing the antileukemic response in B-cell precursor acute lymphoblastic leukemia [169]. Recently, agonistic anti-CD27 antibodies has shown to be effective as monotherapy in reducing the outgrowth of experimental lung metastases and established subcutaneous melanoma tumors *in vivo* [170]. In addition to CD70 agonists, the soluble form of CD70 has been evaluated as powerful adjuvant in a glioblastoma model [171]. The aberrant expression of CD70 in a broad range of hematological malignancies and in some solid tumors has led to the development of CD70-specific T cells, having a CAR receptor consisting of CD27 fused to the CD3- chain. Recently, adoptively transferred CD70-specific T cells have been found to induce regression of established murine xenografts through the recognition of CD70-expressing tumor cells [172].

3.6. GITRL:GITR. Glucocorticoid-induced TNFR-related protein (GITR) is a costimulatory receptor expressed on activated T cells and, constitutively, on Tregs [173]. Its ligand GITRL is expressed at low levels on APCs, but it gets induced following TLR stimulation [173]. Several studies have reported that GITR signaling promotes the proliferation of naïve T cells and cytokines production through the recruitment of TRAF proteins and the activation of downstream pathways [174]. Moreover, one of the first described GITR function was the ability to protect T cells from activation-induced cell death. Controversial data have been reported about the regulation of Treg functions by GITR. In fact, experimental evidences suggest both an inhibitor and a stimulatory role for GITR [174]. The modulation of GITR pathway is an intriguing therapeutic possibility.

The treatment with GITR-expressing adenovirus vector has been shown to be able to induce T-cell response and to reduce tumor size in mice inoculated with B16 tumor cells [175]. Nishikawa et al. reported that triggering GITR through GITRL-expressing plasmid resulted in the inhibition of tumor growth in a CMS5 sarcoma model. The protection of $CD8^+$ T cell against Treg-mediated suppression was also observed by the authors [176]. The use of GITR agonistic antibody (clone DTA-1) is also effective in stimulating antitumor immunity *in vivo* [174]. Recently, Zhou et al. suggested that the antitumor effect of anti-GITR antibody was dependant on its ability to positive costimulate T cells rather than to suppress Treg functions, but the question is still debated [177]. Last year, a clinical study of an agonist anti-GITR antibody (TRX518) in melanoma was started but the trial was put on hold in March because of a major business setback of the company that makes the antibody (Table 2) [99].

3.7. CD30L:CD30. CD30 receptor is an inducible costimulatory receptor expressed on activated and memory T cells following TCR/CD28 or IL-4 stimulation [178]. The ligand of CD30 is CD30L, which is expressed on activated T cells, as well as on macrophages, dendritic cells, and B cells [178]. CD30L/CD30 signaling seems to be involved in Th1 and Th2 cell responses and plays a critical role in Th17 differentiation [178]. The costimulatory signal provided by CD30L:CD30 is not yet at all clear, but it seems to be involved in the peripheral costimulation, mainly supporting T-cell survival, with overlapping features of OX-40 and 4-1BB pathways [167]. Due to the expression of CD30 on all malignant Hodgkin and Reed-Sternberg cells (HRS), this receptor represents an important target for the immunotherapy of hematological malignancies. SGN-35 (brentuximab vedotin) is an anti-CD30 antibody that has been modified by the addition of a dipeptide linker to permit attachment of microtubule polymerization monomethylauristatin E (MMAE) (Table 2) [179]. SGN-35 has been evaluated in phase I dose-escalation study in 45 patients with relapsed or refractory CD30-positive hematologic malignancies and the maximum tolerated dose was determined to be 1.8 mg/kg [180]. In a pivotal phase II study of SGN-35, 102 patients with relapsed or refractory Hodgkin lymphoma were treated with 1.8 mg/kg dose of SGN-35 every three weeks. A reduction in tumor volume was observed in 95% of patients and the overall response rate (ORR) was 75% [181]. The efficacy of SGN-35 has been also evaluated in a phase II single-arm study in 58 patients with anaplastic large cell lymphoma. The authors reported that the ORR was 86% [182]. A multicenter randomized phase III trial of SGN-35 (AETHERA) in posttransplant classical Hodgkin lymphoma patients at high risk for recurrence was started in April 2010 and it should be completed in June 2013. In the light of these impressive result, the FDA approved SGN-35 for the treatment of Hodgkin lymphoma in August 2011. The high efficacy of this antibody-drug conjugate could be due to the fact that cytotoxic effect of MMAE targets not only CD30-expressing HRS cells, but also the immune suppressive Tregs present in the tumor microenvironment because of a bystander effect; moreover, SGN-35 delivers itself an additional apoptotic signal, mainly in anaplastic large cell lymphoma cells [179].

4. Conclusions

Improving the knowledge of T costimulatory and coinhibitory pathways reached over the past decade shed light on the central roles that these molecules play in the generation of an effective immune response. Many tumors escape from immune surveillance through the downregulation of positive costimulatory molecules and the upregulation of coinhibitory signals. Blockade of coinhibitory pathway on the one hand and the stimulation of the positive signals on the other hand have been found to enhance antitumoral immunity, both alone and in combination with traditional therapy in preclinical and clinical trials. Further studies are necessary to evaluate the safety and the efficacy of these approaches before using them in the clinical practice.

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