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Cancer immunotherapy: activating innate and adaptive immunity through CD40 agonists

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Abstract

INTRODUCTION—CD40 is a promising therapeutic target for cancer immunotherapy. In patients with advanced solid malignancies, CD40 agonists have demonstrated some anti-tumor activity and a manageable toxicity profile. A 2nd generation of CD40 agonists has now been designed with optimized Fc receptor (FcR) binding based on preclinical evidence suggesting a critical role for FcR engagement in defining the potency of CD40 agonists *in vivo*.

AREAS COVERED—We provide a comprehensive review using PubMed and Google Patent databases on the current clinical status of CD40 agonists, strategies for applying CD40 agonists in cancer therapy, and the preclinical data that supports and is guiding the future development of CD40 agonists.

EXPERT COMMENTARY—There is a wealth of preclinical data that provide rationale on several distinct approaches for using CD40 agonists in cancer immunotherapy. This data illustrates the need to strategically combine CD40 agonists with other clinically active treatment regimens in order to realize the full potential of activating CD40 *in vivo*. Thus, critical to the success of this class of immune-oncology drugs, which have the potential to restore both innate and adaptive immunosurveillance, will be the identification of biomarkers for monitoring and predicting responses as well as informing mechanisms of treatment resistance.

Keywords

CD40 agonists; macrophages; T cells; chemotherapy; cancer; clinical trials

1. Introduction

Strategies designed to harness the immune system for the treatment of cancer have recently demonstrated significant benefit for some patients across a wide-range of malignancies. For

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Declaration of Interest

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example, immune checkpoint inhibitors designed to disrupt inhibitory signals received by T cells through CTLA-4 and PD-1 molecules can improve overall survival by producing durable remissions in cancer patients [1,2]. However, across many cancers, the vast majority of patients still do not respond to immune checkpoint inhibition using blocking antibodies to CTLA-4 and PD-1/PD-L1. Thus, ongoing efforts in cancer immunotherapy are now focused on *patient selection* (i.e. identifying patients who are most likely to benefit from a particular immunotherapeutic strategy) and understanding *mechanisms of resistance* (i.e. defining the mechanisms that underlie treatment resistance in order to inform the optimization and selection of an immunotherapeutic approach for a patient) [3].

Successful cancer immunotherapy relies on an alignment of the innate and adaptive arms of the immune system. A key molecule involved in bridging innate and adaptive immunity is CD40, a member of the TNF receptor superfamily. Within the innate immune system, CD40 is expressed on antigen presenting cells (APCs) including subsets of monocytes, macrophages, and dendritic cells. CD40 is also expressed by B cells and platelets as well as some non-hematopoeitic cell types such as fibroblasts, endothelial cells, and smooth muscle cells. Even some tumor cells express CD40 [4–6]. Further, ligation of CD40 *in vivo* has the potential to elicit an array of outcomes from activation of APCs to induction of tumor cell death [6–9].

The ligand for CD40 is CD40 ligand (i.e. CD154) which is expressed on a variety of cell types, including activated CD4 T cells [7–9], activated B cells [10], memory CD8 T cells [11], activated natural killer cells [12], granulocytes [13], endothelial cells [14], smooth muscle cells [14], macrophages [14], and activated platelets [15]. In the late 1990s, the interaction between CD40 on dendritic cells (DCs) and CD40 ligand on activated CD4 T cells was found to be a critical step in “licensing” DCs with the capacity to effectively present antigen and activate antigen-specific CD8 T cells [7–9,16]. Specifically, ligation of CD40 on DCs enhanced the expression of co-stimulatory (e.g. CD80 and CD86) and major histocompatibility (MHC) molecules, induced the release of immunostimulatory cytokines, and activated antigen presentation machinery. The importance of CD40 in tumor immunity was subsequently demonstrated in several landmark studies where administration of an agonistic antibody directed against CD40 produced protective T cell immunity in murine models of cancer [17–19]. This early biology laid the foundation for the development of clinical grade CD40 agonists that are now under active investigation in the clinic (Figure 1).

2. Designing a potent CD40 agonist

Several approaches have been investigated to activate the CD40 pathway in humans: (i) recombinant human CD40 ligand, (ii) CD40 ligand gene therapy, and (iii) agonistic CD40 antibodies. Each strategy has produced promising clinical activity in early phase studies. For recombinant human CD40 ligand (rhuCD40L), a Phase I study in patients with advanced solid tumors and non-Hodgkin lymphoma investigated subcutaneous dosing of rhuCD40L for five consecutive days repeated every 4–6 weeks in the absence of progressive disease or organ toxicity [20]. Of 32 patients treated, two (6%) developed a partial response on study with four additional patients (16%) demonstrating stable disease lasting at least four months. For CD40 ligand gene therapy (AdCD40L) using adenoviral vectors expressing CD40

ligand, one study reported on eight patients with bladder carcinoma undergoing cystectomy for invasive disease who were treated with AdCD40L instillation into the bladder [21]. Treatment was found to be generally well-tolerated and produced evidence of immune activation, as detected on biopsy and seen by increased infiltration of T cells and expression of IFN-gamma. A second study evaluated intratumoral administration of AdCD40L in 15 patients with metastatic malignant melanoma who were treated with four weekly injections [22]. Nine of the patients also received treatment in combination with low dose cyclophosphamide. While no objective responses were seen on radiographic imaging, metabolic responses detected on FDG-PET imaging were observed in local and distant lesions with evidence of increased T cell infiltration seen on post-treatment biopsies compared to baseline. Together, these clinical studies demonstrate the prospect of using CD40 ligand-based strategies to activate CD40 in patients for cancer therapy.

The most advanced clinical approach to date for activating CD40 *in vivo* has involved the use of agonistic CD40 monoclonal antibodies. The first report of an agonistic CD40 antibody in patients with advanced solid malignancies investigated CP-870,893, a fully human and selective CD40 agonist monoclonal antibody (mAb) that was designed with minimal Fc receptor binding activity based on its IgG2 isotype [23]. The most common adverse event with CP-870,893 treatment was grade 1–2 cytokine release syndrome manifested by chills, fever and rigors within minutes to hours after infusion. Treatment was also associated with transient decreases in monocytes, B cells, and platelets as well as increases in liver function tests and D-dimer levels. These pharmacodynamics effects of a CD40 agonist, including hepatic injury and leukocyte trafficking from the peripheral blood, have also been reproduced in preclinical models [24–26], although the precise mechanisms underlying this biology remain ill-defined. Nonetheless, this first-in-human study of a CD40 agonist in patients with advanced cancer demonstrated safety as well as promising clinical activity with a response rate of 14%.

2.1. Fc modification

Since the first clinical report of an anti-CD40 agonist, several other agonists have now been developed and are under active investigation in patients with advanced malignancies (Table 1). These 2nd generation CD40 agonists have been engineered with an IgG1 Fc domain to facilitate enhanced Fc gamma receptor (FcγR) interactions based on findings that increased Fc binding affinity to FcγRIIB enhances the potency of a CD40 agonist in murine models through crosslinking [27,28]. These agonists contrast CP-870,893, which has an IgG2 Fc domain, and thus a low binding affinity to human FcγRs. Recently, antibodies with an IgG2 Fc domain have been found to mediate FcγR-independent agonistic activity that is conferred by the unique hinge properties of this isotype [29]. However, for CP-870,893, the Fc domain of the antibody and FcR crosslinking are not be required for CD40 stimulation [30]. The precise mechanism underlying this finding is unclear but could be explained by binding of CP-870,893 to a unique epitope on human CD40 that produces potent signaling activity.

Recent work has shown that engineering CP-870,893 with an IgG1 Fc domain, to enhance binding to FcγRIIB, can improve the potency of CP-870,893 as measured by its ability to invoke antigen-specific T cell immunity in a novel humanized mouse model [31]. FcγRIIB

is the only inhibitory Fc receptor and variations in the gene encoding this protein have long been associated with susceptibility to autoimmune disease [32]. While FcγRIIB polymorphisms are known [32], whether these variations will have therapeutic implications for the translation of IgG1-modified CD40 agonists is presently unclear. In addition, the enhanced potency of IgG1 CD40 agonists seen in murine models is associated with an increased capacity to produce transient thrombocytopenia[31]. Reducing the dose of the Fc-modified CD40 agonist, though, was found to diminish the level of thrombocytopenia while still maintaining improved agonist activity compared to the IgG2 isotype [31].

The requirement for Fc receptor engagement *in vivo* for anti-CD40 efficacy is not absolute, as suggested by the activity of CP-870,893 which displays poor FcR binding. Alternative strategies beyond Fc engineering for enhancing anti-CD40 efficacy, such as chemical crosslinking of CD40 antibodies, have also shown Fc-independent activity in murine models [33]. In addition, the requirement for the inhibitory FcγRIIB for *in vivo* activity of IgG1 anti-CD40 antibodies is not definite. For example, activatory FcRs induced by a TLR3 agonist can restore the *in vivo* activity of an IgG1 CD40 agonist that is otherwise lost in hosts lacking FcγRIIB [33]. This finding suggests that the role of the FcR for anti-CD40 activity is to provide *in vivo* cross-linking. Clinical studies investigating IgG1 CD40 agonists are underway and are expected to provide further insight into the role of Fc modification on CD40 agonist-induced toxicity and efficacy in patients with advanced malignancies.

2.2. Clinical grade antibodies targeting CD40

Over the past decade, several clinical grade antibodies that target CD40 have been developed. Each of these agents is distinct with unique properties defined by (i) binding affinity to CD40, (ii) isotype, (iii) requirement for cross-linking for activity, and (iv) ability to block CD40 ligand binding. These clinical grade antibodies include:

1. **CP-870,893** is an anti-CD40 IgG2 antibody with poor FcR binding that does not block CD40 ligand interaction with CD40; can mediate CD40 stimulation in the absence of cross-linking [30]; and has a binding affinity (Kd) of 3.48×10^{-10}M [34].
2. **APX005** is an IgG1 antibody recognizing CD40 that blocks CD40 ligand binding; shows enhanced activity *in vitro* with cross-linking; and has a Kd of 9.6×10^{-10}M [35].
3. **ADC-1013** is an IgG1 antibody that recognizes CD40 with high binding affinity (Kd 1×10^{-11}M) even under acidic conditions (pH 5.4) with activity dependent on FcR binding and cross-linking [36].
4. **Dacetuzumab (also called SGN-40 and formerly SGN-14)** is a humanized IgG1 antibody recognizing CD40 with a Kd of 1×10^{-9}M. Dacetuzumab is a partial agonist that shows weak activity in stimulating B cell proliferation; displays potent anti-proliferative and pro-apoptotic properties against B cell lymphoma lines; and enhances CD40 ligand binding to CD40 [37,38].

5. **SEA-CD40** is a non-fucosylated humanized IgG1 anti-CD40 antibody derived from dacetuzumab (SGN-40) with a Kd of 1×10^{-9}M. SEA-CD40 shows improved agonist activity due to enhanced binding to FcγRIIIa [39,40].

6. **ChiLob 7/4** is a chimeric IgG1 antibody with a Kd of 2×10^{-10}M [41] that requires cross-linking for CD40 stimulation in antigen-presenting cells [42].

7. **Lucatumumab (HCD122)** is a fully humanized IgG1 anti-CD40 antagonist that blocks CD40 ligand engagement with CD40. Lucatumumab has a Kd of 5×10^{-10}M and does not display agonistic activity but induces tumor cell death via antibody-dependent cell-mediated cytotoxicity and opsonization [43].

2.3. Toxicity

The use of CD40 agonists in patients with cancer has been associated with several toxicities. For example, treatment with CP-870,893 produces a cytokine release syndrome (CRS) in the majority of patients that is characterized by fever, rigors, and chills. This CRS occurs within minutes to hours after treatment and was the dose-limiting toxicity for CP-870,893 in its early phase dose-finding study [23]. Evidence of hepatotoxicity is also commonly observed with CD40 agonists. Transient elevations in transaminases can be detected within 24 hours of treatment and persist for several weeks before resolution. In preclinical models, this hepatotoxicity has been found to be dependent on CD40-expressing hematopoietic cells, specifically $CD11b^{+}$ $Gr\text{-}1^{+}$ myeloid cells [24]. In addition, the liver injury induced by an agonistic CD40 antibody is dependent, at least in part, on NADPH oxidase 2 and reactive oxygen species [24]. Preclinical models have suggested that the degree of toxicity associated with systemic immune activation may also be influenced by age. For example, when a CD40 agonist is combined with IL-2 immunotherapy, lethal hepatotoxicity is observed with increasing age [44]. This mortality and the associated pathology seen in the liver, as well as lung and gut, were associated with macrophage-dependent induction of proinflammatory cytokines including IL-6, TNF-α, and IFN-γ. In particular, TNF-α was a major mediator of liver injury and mortality produced with anti-CD40/IL-2 treatment [44]. This finding illustrates the potential role of aging in defining toxicity to CD40 immunotherapy.

Thromboembolic events have also been seen with CP-870,893 in several patients, but the relationship of these events to anti-CD40 treatment has been confounded by the increased risk of this adverse event with cancer burden. Autoimmune events, including dermatitis, colitis, hypophysitis and thyroiditis, have not been seen with CD40 antibodies. However, additional toxicities reported with CD40 antibodies do include infusion reactions, noninfectious inflammatory eye disorders (seen specifically with dacetuzumab [45]), anemia, thrombocytopenia, and pleural effusion [23,45–48].

Strategies to ameliorate toxicity associated with a CD40 agonist have been investigated by several groups. One approach involves peritumoral injection. In an immunogenic model of bladder cancer, local peritumoral injection of an agonistic CD40 antibody was found to effectively elicit a tumor-specific T cell response at reduced doses compared to intravenous injection [49]. In addition, biodistribution of CD40 antibodies to the liver was decreased with local compared to systemic injection [49]. Slow-release delivery has also been studied

as a strategy to induce immune activation without systemic toxicity. For example, administration of an agonistic CD40 antibody locally in mineral oil Montanide ISA 51 can induce local DC activation leading to tumor-specific T cell immune responses with decreased systemic toxicity compared to systemic administration of an anti-CD40 antibody [50]. However, tumor-specific T cell responses induced with this local injection approach were restricted to antigens presented in the tumor-draining area [50]. Finally, administration of TNF blocking antibodies in combination with anti-CD40/IL-2 immunotherapy has also been shown to lessen treatment-induced hepatotoxicity observed in aged mice while maintaining T cell-dependent anti-tumor activity [44].

In addition to toxicity, CD40 agonists may also induce undesired biology. For example, triggering of CD40 on endothelial cells has been shown to stimulate the angiogenic process and to promote tumor growth [44], thus identifying a potentially undesired site of CD40 activation. CD40 activation has also been implicated in the transformation of primary B cells [51] as well as lymphomagenesis [52]. However, evidence in patients treated with CD40 agonists for these potential pro-tumorigenic events has not been reported to date. Finally, administering chemotherapy within 48 hours after a CD40 agonist can produce lethal toxicity in mouse models of cancer [26]. This toxicity, though, can be avoided by delaying administration of chemotherapy to five days after a CD40 agonist [26].

3. Preclinical modeling of CD40 agonists

Over the past two decades, CD40 agonists have been investigated pre-clinically with studies revealing several approaches for their incorporation into cancer immunotherapy. Early work demonstrated the potential of a CD40 agonist to “license” antigen-presenting cells with the capacity to stimulate potent anti-tumor T cell immunity in several mouse models of cancer [17–19]. Subsequent studies then revealed that toll-like receptor (TLR) agonists could significantly improve the T cell stimulatory capacity of CD40 antibodies. For TLR3, 4, 7, and 9, this effect was dependent on type I IFN signaling [53]. It was then shown that co-administration of a CD40 antibody with a TLR7 agonist and peptide antigen could induce a marked expansion of antigen-specific CD8+ T cells with enhanced cytolytic activity capable of delaying the progression of melanoma in a lung metastasis model [54]. TLR7 agonism was also found to reverse hepatotoxicity seen with anti-CD40 treatment [54].

The ability to enhance T cell priming versus boosting memory T cell responses with CD40 stimuli can be influenced by TLR agonists. For example, combining CD40 antibodies with a TLR3 (Poly I:C) or TLR9 (CpG) agonist significantly enhances the priming effect of CD40 stimuli in combination with a peptide vaccine [55]. However, only the TLR3 agonist with peptide and anti-CD40 effectively boosted tumor-specific CD8 T cell immunity which occurred independently of CD4+ T cells [55,56]. In nonhuman primates, the combination of a human anti-CD40 antibody and a TLR3 agonist (Poly IC:LC) with a peptide antigen also produced potent antigen-specific T cell activation particularly within the lung [57].

The mechanism by which a TLR agonist synergizes with anti-CD40 is due at least in part to the induction of CD70 on DCs which is critical for CD8 T cell priming [58,59]. This induction of CD70 on DCs can also be achieved by combining type I interferons or natural

killer (NK) T cell ligands, including α-galactosylceramide (αGalCer) or αC-GalCer, with a CD40 agonist [60]. Together, these findings illustrate the potential of combining CD40 stimuli with TLR agonists, type I interferons, or NKT ligands to elicit potent antigen-specific anti-tumor T cell immunity.

CD40 agonists have also been combined with IL-2 immunotherapy as a strategy to modulate immunosuppression within the tumor microenvironment and to induce tumor-specific T cell immunity. In an orthotopic mouse model of metastatic renal cell carcinoma, this combinatorial treatment approach induced complete regression of metastatic tumors and potent T cell dependent immunity [61]. This anti-tumor activity was associated with increased infiltration of CD8 T cells and NK cells with a concomitant IFN-gamma and Fas-dependent reduction of CD4+ Foxp3+ regulatory T cells as well as suppressive myeloid cells within the tumor microenvironment [62,63]. In addition to this effect on T cells and immunoregulatory cell populations, IL-2/anti-CD40 immunotherapy can stimulate macrophages to inhibit lung metastasis. This effect requires macrophage-dependent nitric oxide synthase expression and subsequent production of nitric oxide to downregulate within primary tumors the expression of matrix metalloproteinases (MMP) known to be key mediators of the metastatic process, specifically MMP-2 and MMP-9 [5]. Thus, combining CD40 stimuli with IL-2 immunotherapy can promote enhanced innate and adaptive immunosurveillance in cancer and in doing so, impact both primary and metastatic disease.

CD40 agonists might also be used to improve the efficacy of adoptive T cell therapies. In a mouse model of melanoma, the combination of IL-2/anti-CD40 with adoptive cell transfer (ACT) of tumor-specific activated T cells recognizing gp100 produced strong anti-tumor activity that was more effective than either ACT of activated T cells or IL-2/anti-CD40 alone [64]. This effect was associated with enhanced *in vivo* expansion of adoptively transferred CD8+ T cells and dependent on CD40 expression by bone marrow derived cells, IL-12 production, and CD80/86 expression but not CD4 T cells, B cells or CD11c+ cells. In addition, the expansion of adoptively transferred T cells was not dependent on antigen cross-presentation. This finding is consistent with the potential of a CD40 agonist to induce an antigen-nonspecific expansion of memory CD8+ T cells [65]. Thus, this data supports a potential role for CD40 agonists in conditioning the host for enhancing the activity of adoptively transferred activated T cells.

4. Clinical application of CD40 agonists to cancer therapy

The use of CD40 agonists for inducing anti-tumor immune responses has primarily focused on strategies that ignite T cell-dependent anti-tumor immunity as described above. However, CD40 agonists have also been found to directly induce malignant cell apoptosis and to modulate innate immune surveillance with significant therapeutic implications. Thus, three major approaches for using CD40 agonists in cancer have emerged: (i) direct anti-tumor activity, (ii) induction of T cell immunity, and (iii) activation of innate immunosurveillance). Here, we discuss the clinical status of each of these strategies and the preclinical data that supports the rationale of ongoing investigations.

4.1. Direct anti-tumor activity

CD40 is expressed on most B cell malignancies, including multiple myeloma, non-Hodgkin's lymphoma, and chronic lymphocytic leukemia [66,67]. In addition, CD40 has been detected on many solid malignancies, including melanoma, ovarian, bladder, breast, and cervical cancer among others [6,68]. Thus, CD40 expression on malignant cells makes it a potential therapeutic target.

Although CD40 activation has been implicated in lymphomagenesis and the transformation of primary B cells [51,52], CD40 signaling in malignant cells, including many B cell lymphomas and solid malignancies, can produce growth arrest and apoptosis [6]. This effect has been seen with both CD40 ligand [69] and anti-CD40 antibodies [70], and is due at least in part to upregulation of pro-apoptotic proteins such as Bcl-2-associated X protein (BAX) [71].

The potential of CD40 to serve as a therapeutic target has been seen in B cell lymphomas. For example, lucatumumab, a CD40 antagonistic monoclonal antibody, produced regressions in a subset of patients with advanced Hodgkin lymphoma and non-Hodgkin lymphoma [72]. Similarly, in high grade B cell lymphoma, dacetuzumab (SGN-40), which is a weak anti-CD40 agonist, showed potent anti-proliferative and pro-apoptotic activity against malignant cells *in vitro* and *in vivo* [70]. This direct cytotoxic effect of dacetuzumab may be due at least in part to antibody-dependent cellular phagocytosis mediated by macrophages [73]. In patients with refractory or recurrent B-cell lymphoma, dacetuzumab produced a response rate of 12% with one complete response among 50 treated patients [45]. Subsequent studies investigating the sensitivity of non-Hodgkin lymphoma cell lines to CD40 stimulation with dacetuzumab have identified a correlation with intrinsic DNA damage, increased proliferative rate, and expression of BCL6 that predict treatment response [37]. In addition, a pre-existing activation of the CD40 pathway in malignant cells was found to strongly correlate with resistance to the direct cytotoxic effects of CD40 stimulation and was associated with clinical response in patients with diffuse large B cell lymphoma treated with dacetuzumab where tumor shrinkage was seen in 21 of 57 (37%) patients [37]. Fc-engineering to enhance binding of CD40 antibodies to FcγRs may also enhance the direct cytotoxic activity of this class of drugs by improving their ability to stimulate antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis by macrophages [74]. In addition to producing direct anti-tumor activity, ligation of CD40 on malignant cells can induce upregulation of MHC molecules, the secretion of multiple soluble factors (e.g. IL-6, IL-8, TNF-α and GM-CSF), and also enhance malignant cell susceptibility to T cell lysis, as seen in melanoma [75]. Thus, CD40 expression on malignant cells is a promising therapeutic target.

4.2. Activating adaptive immunity

Studies investigating CD40 agonists were initially conducted in immunogenic tumor models and showed that a CD40 agonist could, even by itself, invoke potent T cell dependent anti-tumor immunity leading to cures [17]. However, a critical component to the success of this approach was the presence of tumor antigen, which is necessary for CD40-activated APCs to induce antigen-specific T cell immunity *in vivo* [18,19]. This biology underlies the

hypothesis for ongoing investigations that are combining CD40 agonists with vaccines and chemotherapy. For example, because chemotherapy can elicit an immunogenic form of tumor cell death [76], chemotherapy may induce the release of tumor antigens that would then be phagocytosed by APCs (Figure 2A). With subsequent ligation of the CD40 molecule on APCs, tumor antigenic peptides would then be presented in the context of MHC molecules for antigen-specific T cell stimulation. Thus, this hypothesis, which is supported by preclinical models [77,78], suggests that the sequence of combining CD40 agonists with chemotherapy for activating T cell immunity is critical such that chemotherapy should be administered prior to a CD40 agonist.

The use of chemotherapy to enhance the T cell-stimulatory capacity of a CD40 agonist has been investigated in three clinical studies (Table 2). In the first study, CP-870,893 was combined with paclitaxel and carboplatin and produced a best overall response rate of 20% across patients with multiple solid tumor malignancies [79]. Patients received chemotherapy on day 0 with CP-870,893 administered on day 2 or 7. However, the timing of CP-870,893 administration after chemotherapy did not appear to impact the response rate. Amongst metastatic melanoma patients, the response rate was 12% with 3 of 28 patients achieving a partial response (PR). This finding, though, is very similar to the best overall response reported for CP-870,893 alone in patients with metastatic melanoma where 27% (4 of 15) of patients achieved a PR [23]. In the second study, CP-870,893 was combined with cisplatin and pemetrexed in patients with malignant pleural mesothelioma [46]. Here, CP-870,893 was administered 7 days after chemotherapy. In this study, treatment was well tolerated, but the objective response rate of 40% (6 of 15 patients) was similar to that expected for chemotherapy alone [80]. In the third study, CP-870,893 was combined with weekly dosing of gemcitabine in patients with chemotherapy naïve advanced pancreatic ductal adenocarcinoma [25,48]. CP-870,893 was administered 2 days after the first dose of chemotherapy during each cycle. This treatment strategy produced a response rate of 24% (5 of 21 patients) which was higher than expected for gemcitabine alone which achieves a historical tumor response rate of 5–10% [81–83]. However, evidence for T cell-dependent anti-tumor activity was not observed. Rather, preclinical findings supported a role for CD40 stimulation in invoking productive innate immunity with macrophages mediating anti-tumor activity [25].

The lack of benefit seen clinically with delivering chemotherapy prior to a CD40 agonist may reflect the presence of additional immune suppressive mechanisms orchestrated by developing tumors. Consistent with this idea, in a mouse model of spontaneous pancreatic carcinoma, macrophages residing outside of the tumor microenvironment have been shown to regulate the potential of gemcitabine and a CD40 agonist to elicit productive T cell anti-tumor immunity [78]. This finding implies a role for additional mechanisms beyond CD40 that are critical for T cell priming and infiltration into poorly immunogenic cancers. The mechanism of immune suppression mediated by this population of macrophages, though, is currently unknown.

To unleash the therapeutic potential of a CD40 agonist, combination studies are now being explored with more potent chemotherapeutic regimens under the premise that this will improve antigen release, shift the innate immune reaction to cancer from

immunosuppressive to immunostimulatory, or both [84]. An ongoing neoadjuvant clinical study combining CP-870,893 with gemcitabine and nab-paclitaxel in patients with surgically resectable pancreatic carcinoma may provide insight into this prospect of using chemotherapy in combination with a CD40 agonist to induce T cell dependent anti-tumor immunity in a poorly immunogenic tumor (NC02588443). In addition, CD40 agonists are being combined with inhibitors of immune checkpoint molecules such as CTLA-4 (NCT01103635) and PD-1/PD-L1 (NCT02304393, NCT2706353) blocking antibodies. A phase I study investigating CP-870,893 in combination with tremelimumab, a fully human IgG2 mAb targeting CTLA-4, showed clinical activity with a response rate of 27.3% in patients with metastatic melanoma [85]. Further studies, though, are required to discern whether this activity is superior to that previously seen with single agent treatment of CP-870,893 (RR 27%) [23] or tremelimumab (11%) [86] in patients with metastatic melanoma. Certainly, in immunogenic models of cancer, blockade of CTLA-4 and PD-1/PD-L1 has enhanced the therapeutic potential of a CD40 agonist [87]. However, in poorly immunogenic spontaneously arising tumors, thus far this strategy has been met with marked resistance [88]. Nonetheless, the prospect of using a CD40 agonist to stimulate T cell dependent anti-tumor immunity will likely require additional interventions (e.g. TLR agonists or cytokine-based immunotherapy as discussed above) that are aimed at activating key immune pathways and disrupting critical inhibitory pathways that together regulate the T cell stimulatory capacity of a CD40 agonist.

4.3. Activating innate immunity

The development of CD40 agonists for cancer immunotherapy has almost exclusively focused on the potential of CD40 to bridge innate and adaptive immunity for induction of productive anti-tumor T cell immune responses. However, CD40 agonists have also been found to redirect tumor-infiltrating myeloid cells from pro- to anti-tumor [25,89]. This biology has been seen in several murine models of cancer including spontaneous pancreatic carcinoma, neuroblastoma, and melanoma where treatment with a CD40 agonist activates macrophages with tumoricidal activity [25,26,89]. In a model of pancreatic carcinoma, macrophage activation resulted in rapid degradation of collagen-based cancer fibrosis seen as early as 18 hours after treatment [25,26]. This anti-fibrotic effect was dependent on a subset of $CCR2^{+}$ monocytes that were recruited to tumors via the chemokine CCL2. Tumor-infiltrating monocytes were activated by IFN-γ released systemically in response to CD40 activation and were necessary to shift the profile of matrix metalloproteinases within tumors in favor of fibrosis degradation (Figure 2B). The implications of this anti-fibrotic activity, though, were not initially appreciated as by itself, treatment with a CD40 agonist produced macrophage-dependent tumor regressions albeit transiently [25].

The capacity of a CD40 agonist to modulate fibrosis, which can act as a diffusional barrier to drug delivery [90,91], suggested its potential to also enhance the activity of cytotoxics. To this end, CD40 agonists have been found to condition tumors for enhanced sensitivity to chemotherapy [26]. This finding may explain the anti-tumor activity seen in a Phase I study of patients with advanced pancreatic carcinoma treated with gemcitabine chemotherapy and a CD40 agonist [25,48]. In this study, patients received a first dose of gemcitabine at 48 hours prior to a CD40 agonist, but then also received two additional infusions of

gemcitabine at 5 and 12 days after CD40 treatment. The dose of gemcitabine delivered prior to CD40 treatment was intended to facilitate release of tumor antigen for activation of antigen-specific T cells. However, biopsies of regressing tumors showed no evidence of T cell infiltration to explain tumor responses, which were ultimately concluded to be related to macrophage-dependent anti-tumor activity based on preclinical modeling [25]. In retrospect, this initial dose of gemcitabine prior to a CD40 agonist may have been detrimental due to the myelosuppressive effects of gemcitabine [81,83], which could have limited the full potential of macrophage-dependent anti-tumor activity. In contrast, the timing of gemcitabine administration at 5 days after a CD40 agonist has now been shown to produce enhanced cytotoxic activity in preclinical models [26]. This timing has also been found to be safe in both patients and mice, although delivery of chemotherapy within 48–72 hours after a CD40 agonist is associated with severe hepatotoxicity [26,92]. While the precise mechanism of this toxicity is unclear, proper timing of chemotherapy after a CD40 agonist avoids this adverse event while maintaining therapeutic efficacy [26]. Thus, CD40 agonists may offer a novel strategy for improving outcomes with standard of care cytotoxic chemotherapy.

The finding that CD40 agonists could induce productive immunosurveillance independent of T cells was unexpected, but demonstrates the inherent plasticity of the innate immune response to cancer and its potential to be harnessed for therapy. A major difference, though, between macrophage- and T cell-dependent immunity is in the ability to produce memory responses. Whereas T cells can provide long-lived immunosurveillance, macrophages are more likely to be beneficial for tumor debulking. Nonetheless, strong preclinical rationale support further clinical investigation of CD40 agonists for enhancing tumor sensitivity to cytotoxic therapies and invoking macrophage-dependent anti-tumor immunity.

5. Concluding Remarks

The CD40 pathway is involved in several facets of immune regulation. Expressed on multiple cell types of hematopoietic and non-hematopoietic origin, CD40 is a critical regulator of anti-tumor immunity due to its ability to “license” APCs for priming of antigen-specific T cells and its capacity to redirect tumor-infiltrating myeloid cells with anti-tumor and anti-fibrotic activity. Agonists capable of activating the CD40 pathway have been developed and critical elements to their design have been elucidated – in particular the role of crosslinking for unleashing the potency of a CD40 agonist *in vivo*. Based on this knowledge, several CD40 agonists engineered for enhanced FcR binding have now entered the clinic and are being evaluated. Yet, challenges remain in how to develop CD40 agonists for cancer immunotherapy. In hematological malignancies, CD40 antibodies have been used to induce direct cytotoxic activity and to elicit mechanisms of antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis. In contrast, in solid malignancies the primary focus has been to use CD40 agonists to provoke T cell dependent anti-tumor immunity. While this strategy has produced some efficacy, particularly in patients with metastatic melanoma, many patients do not respond. Preclinical data suggest that this may be due, at least in part, to a subset of macrophages that regulate the capacity of a CD40 agonist to stimulate tumor-specific T cells and the need for additional immune stimuli (e.g. TLR agonists or cytokines including IL-2 and type I interferons) to effectively prime and boost tumor-specific T cell immunity. In contrast, CD40 agonists have recently been shown

to also condition tumors for enhanced sensitivity to chemotherapy. This finding may explain encouraging results seen when chemotherapy was combined with a CD40 agonist in patients with pancreatic carcinoma and deserves further clinical investigation to determine the potential of this strategy. Together, CD40 remains a promising target for cancer immunotherapy that has demonstrated some activity in patients. Building on this experience with rational drug combinations and proper sequencing with cytotoxic chemotherapy will be critical for its ultimate incorporation into the immunotherapy armamentarium.

6. Expert Commentary

CD40 agonists can be potent stimulants of the immune system. However, as single agent therapy, clinical activity has been limited and thus, it has become clear that effective translation of CD40 agonists into cancer therapy will hinge on its combination with other treatments. This will require not only an increased understanding of what makes a CD40 agonist effective *in vivo* but also identification of measurable biomarkers to monitor its activity. Preclinical studies have suggested a role for Fc modification for enhancing the potency of an agonist CD40 mAb. Whether this design strategy will improve the activity of a CD40 agonist in patients, though, still remains to be determined.

CD40 agonists can stimulate both innate and adaptive anti-tumor immunity as well as produce direct cytotoxic activity against CD40 expressing malignant cells. While much attention has focused on how to use CD40 agonists to stimulate T cell immunity, preclinical models have shown that CD40 agonists can also be used to condition tumors for enhanced sensitivity to chemotherapy. Biomarkers to monitor this “conditioning” effect have been identified and can be used to understand the potential of this strategy in patients. In contrast, mechanisms that regulate the capacity of CD40 agonists to induce T cell immunity are still being understood. In both patients and preclinical models, T cell stimulation with CD40 agonists has been most successful in immunogenic tumor models. This finding may suggest a role for CD40 agonists in bolstering existing, but weak, T cell immune responses to cancer and thus, supports a development approach for CD40 agonists that focuses on patient selection to identify those who are most likely to benefit from CD40 immunotherapy. Further, preclinical studies have clearly defined that additional stimuli can enhance the potency of a CD40 agonist for priming and boosting antigen-specific T cell immunity. In addition, CD40 agonists have shown potential in preclinical models for improving the efficacy of adoptive T cell therapies. Moving forward, combining CD40 agonists with additional immune agonists (e.g. TLR agonists) designed to enhance the T cell stimulatory capacity of antigen presenting cells and with adoptive T cell therapy deserves clinical investigation. This combinatorial approach to using a CD40 agonist may also be critical to unleashing T cell immune responses against poorly immunogenic tumors marked by T cell exclusion.

7. Five-year view

Continued investigations into the biology that regulates the capacity of CD40 agonists to invoke T cell immune responses and condition tumors for enhanced responsiveness to cytotoxic therapies will help inform the future development of CD40 agonists. Molecular

profiling and multiplex immunohistochemistry will aid in the identification of biomarkers of both response and resistance to CD40 therapy which will guide the positioning of this unique class of immune oncology drugs. Over the next several years, safety and clinical activity of a 2nd generation of CD40 agonists will be unveiled and in doing so, will create opportunities for strategically-designed treatment combinations aimed at broadening the potential of immunotherapy. It is expected that CD40 agonists will be evaluated in combination with cytotoxic agents, novel immune checkpoint inhibitors, and other immune agonists. Overall, CD40 agonists are strong immune stimulants but are also only one piece of a complex puzzle necessary to restore productive innate and adaptive immune surveillance in cancer.

8. Key Issues

- The CD40 molecule is a member of the TNF receptor superfamily and is expressed by multiple cell types including monocytes, macrophages, dendritic cells, B cells, platelets, fibroblasts, endothelial cells, smooth muscle cells and some malignant epithelial cells.
- The ligand for CD40, CD40 ligand (CD154), is expressed on a variety of cells including activated CD4 T cells, activated B cells, memory CD8 T cells, activated natural killer cells, granulocytes, endothelial cells, smooth muscle cells, and activated platelets. Activation of the CD40 pathway is a critical step in "licensing" antigen presenting cells with the capacity to effectively present antigen and stimulate antigen-specific T cells.
- CD40 targeted therapies include recombinant human CD40 ligand, CD40 ligand gene therapy, and CD40 antibodies.
- CD40 monoclonal antibodies can stimulate innate and adaptive anti-tumor immunity as well as mediate direct cytotoxic activity against malignant cells.
- In early phase clinical studies, CD40 antibodies have been generally well-tolerated and shown promising anti-tumor activity.
- Systemic immune activation elicited by CD40 agonists can produce toxicity that limits dosing. Preclinical models have suggested novel strategies for improving toxicity without impacting efficacy.
- Fc-engineering or TLR activation to enhance FcR binding *in vivo* and chemically cross-linking of CD40 agonists can increase the potency of anti-CD40 antibodies through Fc-dependent and -independent mechanisms, respectively.
- In some preclinical models, chemotherapy administered prior to a CD40 agonist enhances the development of T cell dependent anti-tumor immunity, although this treatment combination has not yet produced similar results in patients.
- Additional immune stimuli (e.g. TLR agonists, IL-2, and type I IFN) can improve the capacity of CD40 agonists to prime and boost antigen-specific T cell immune responses.

- CD40 agonists elicit a systemic immune reaction that can induce tumor-infiltrating myeloid cells with anti-tumor and anti-fibrotic properties.
- CD40 agonists can be used to condition tumors for enhanced chemotherapy efficacy supporting a role for CD40 agonists delivered prior to chemotherapy.
- Several clinical grade CD40 agonists are being evaluated in early phase clinical trials.

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* Article of interest

** Article of considerable interest

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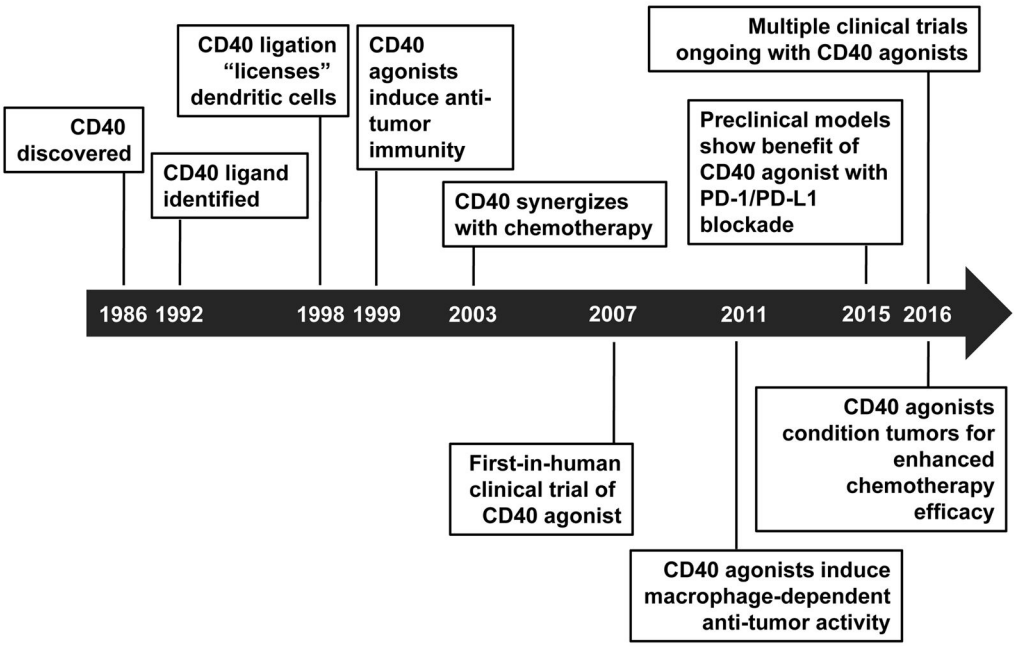

Figure 1. Milestones in the history of CD40 as a target for cancer immunotherapy

The timeline depicts some of the pivotal milestones in the study of CD40 as a target for cancer immunotherapy. 1990 – CD40 discovered [93]. 1992 – CD40 ligand identified [94,95]. 1998 – CD40 ligation "licenses" dendritic cells [7–9,16]. 1999 – CD40 agonists induce anti-tumor immunity [17–19]. 2003 – CD40 synergizes with chemotherapy [77]. 2007 – First-in-human clinical trial of CD40 agonist [23]. 2011 – CD40 agonists induce macrophage-dependent anti-tumor immunity [25]. 2015 – Preclinical models show benefit of CD40 agonist with PD-1/PD-L1 blockade [87]. 2016 – CD40 agonists "condition" tumors for enhanced chemotherapy efficacy [26]. 2016 –Multiple clinical trials ongoing with CD40 agonists.

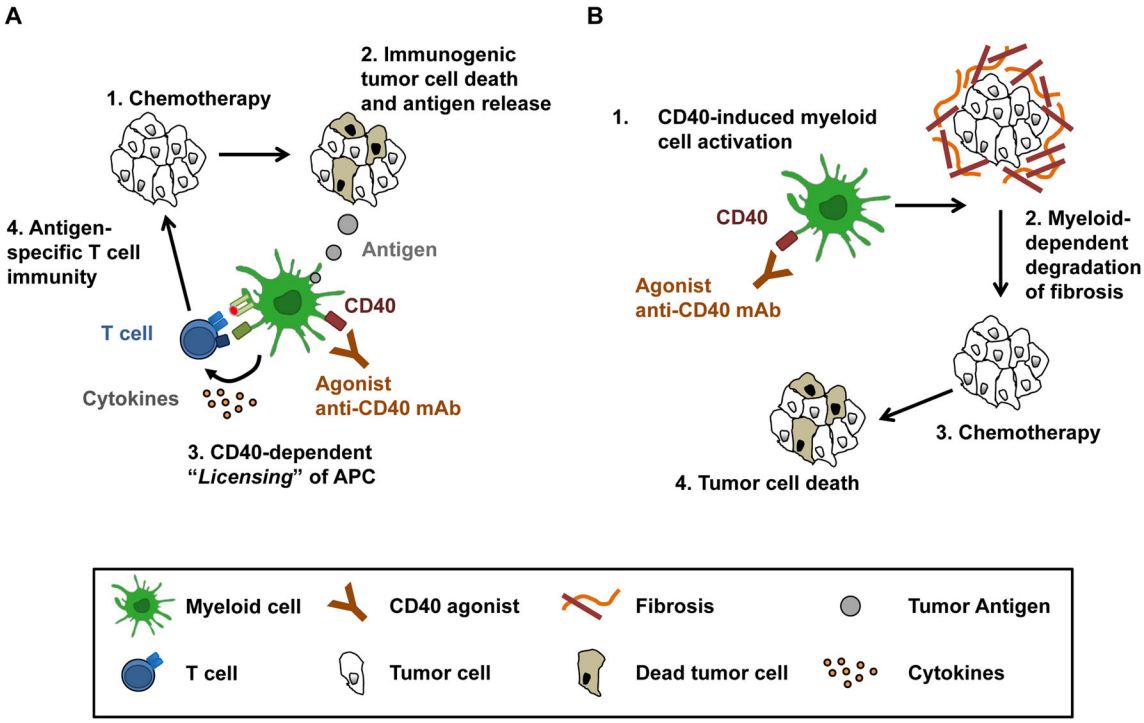

Figure 2. Strategies for applying CD40 agonists in cancer therapy

A. *Stimulating T cell immunity.* Treatment with chemotherapy (step 1) induces an immunogenic form of tumor cell death with the release of tumor cell antigens (step 2) that are phagocytosed by APCs, such as macrophages and dendritic cells. APCs are then "licensed" by a CD40 agonist (step 3) to present tumor antigen and stimulate tumor specific T cells (step 4). **B.** *Activating innate immunity.* Treatment with a CD40 agonist stimulates tumor-infiltrating myeloid cells (step 1) to induce degradation of tumor-associated fibrosis (step 2). Loss of fibrosis renders the tumor more susceptible to chemotherapy (step 3) resulting in tumor cell death (step 4).

Table 1

CD40 agonists and current clinical status

CD40 agonist	Isotype	Active Clinical Studies
RO7009789 (formerly CP-870,893)	Fully human IgG2 agonist	• CD40 + anti-PDL1 (NCT02304393) • CD40 + anti-Ang2/VEGF (NCT02665416) • CD40 + anti-CSF1R (NCT02760797) • Gemcitabine/nab-Paclitaxel + CD40 (NCT02588443)
APX005M	Humanized rabbit IgG1 agonist	• CD40 + anti-PD1 (NCT2706353) • CD40 alone (NCT02482168)
ADC-1013	Fully human IgG1 agonist	• CD40 alone (NCT02379741)
Chi Lob 7/4	Chimeric IgG1 agonist	• No active studies
SEA-CD40	Non-fucosylated humanized IgG1 agonist	• CD40 alone (NCT02376699)

Table 2

History of CD40 agonist clinical trials

Drugs	Phase	# of patients	Tumor type	Efficacy	ClinicalTrials.gov Identifier	References
CP-870,893 (single dose)	1	29	Advanced solid tumors	BORR: 14%	NCT02225002	[15,42]
CP-870,893 (weekly dosing)	1	27	Advanced solid tumors	BORR: 0%		[43]
CP-870,893 + Carboplatin/Paclitaxel	1	30	Advanced solid tumors	BORR: 20%	NCT00607048	[26]
CP-870,893 + Cisplatin/Pemetrexed	1	15	Mesothelioma	BORR: 40%		[27]
CP-870,893 + Gemcitabine	1	21	Pancreas cancer	BORR: 24%	NCT00711191	[17,29]
CP-870,893 + Tremelimumab	1	24	Melanoma	BORR: 27.3%	NCT01103635	[34]
CP-870,893 + poly IC:LC + peptide vaccine	1		Melanoma	Not reported	NCT01008527	
ChiLob 7/4 (weekly dosing)	1	21	Advanced solid tumors	BORR: 0%	NCT01561911	[44]

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