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Review

The Janus faces of CD40 in cancer

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ABSTRACT

CD40 is a TNF receptor family member that is widely recognized for its prominent role in immune regulation and homeostasis. Expression of CD40 is not restricted to normal lymphoid cells but is also evident in the majority of haemopoietic and epithelial malignancies where it has been implicated in oncogenic events. Accumulating evidence, however, suggests that the CD40 pathway can be exploited for cancer therapy by virtue of its ability to stimulate the host anti-tumor immune response, normalize the tumor microenvironment and directly suppress the growth of CD40-positive tumors. Here, we provide an overview of the multifaceted functions of the CD40 pathway in cancer and its emerging role in the treatment of malignancy.

1. Introduction

The CD40 pathway has attracted significant attention among immunologists because of its prominent role in orchestrating the humoral and cell-mediated immune response [1]. Thus, the interaction of CD40 on the surface of B lymphocytes with the CD40 ligand (CD154), which is predominantly expressed by activated T cells, is critical for the induction of adaptive immunity by promoting the proliferation and differentiation of B lymphocytes into immunoglobulin-producing plasma cells. The CD40–CD154 interactions are also important in the activation of macrophages and the amplification of the innate immune response to intracellular and extracellular pathogens. In addition, CD154 is one of the most potent stimulators of dendritic cells (DCs) which engulf debris and dying cells in tissues and influence T-cell priming and maintenance of T cell tolerance to autoantigens [2]. Disruption of the CD40 pathway would therefore be predicted to confer deleterious effects on immune function. Indeed, mutations in the *CD154* gene in humans results in the X-linked hyper IgM syndrome, a severe immune deficiency that is clinically manifested by recurrent viral and bacterial infections and early lethality [3]. However, the expression of CD40 is not restricted to immune cells but extends to a variety of other normal cell types, including fibroblasts, neuronal, epithelial and endothelial cells [4–7] and this widespread expression indicates that CD40 may play a broader role in human physiology and disease pathogenesis.

The pleiotropic functions and prevalent expression of CD40 have also sparked an immense interest in the mechanisms of CD40 signal transduction. Structurally, CD40 comprises a 277 amino acid protein with a large 193 amino acid extracellular domain, a 22 amino acid transmembrane region and a short 62 amino acid cytoplasmic C-terminus. The cytoplasmic tail of CD40 lacks intrinsic kinase activity and signals largely through the ligand-dependent recruitment of adaptor proteins of the TNF receptor-associated factor (TRAF) family [8]. TRAFs not only bridge CD40 to intracellular signalling components but also operate as E3 ubiquitin ligases to activate proximal protein kinases that lead to the engagement of the c-Jun N-terminal kinase (JNK), extracellular-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways, the phosphatidylinositol 3-kinase (PI3K) cascade and the transcription factors nuclear factor-κB (NF-κB) and signal transducer and activator of transcription (STAT) [8,9]. These pathways act in concert to regulate many of the reported activities of CD40 in a cell type and microenvironment dependent manner. Here, we provide an overview of the tumor growth-regulating properties of CD40 and its emerging role in the treatment of malignancy.

2. Does chronic activation of the CD40 pathway contribute to the pathogenesis of cancer?

Deregulated production of CD154 leading to chronic engagement of the CD40 pathway has been implicated in a number of human pathologies, including atherosclerosis and autoimmunity [10,11]. As the majority of haemopoietic and nearly 75% of all epithelial malignancies examined so far express high levels of CD40, this pathway has also been scrutinized for its involvement in the pathogenesis of cancer. Studies in non-Hodgkin lymphoma, chronic

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lymphocytic leukaemia and Burkitt lymphoma have shown that these cells produce and exploit CD154 in autocrine mode to sustain their proliferation and survival, largely through the constitutive engagement of the NF-κB pathway [12,13]. A similar scenario may occur in solid tumors such as melanoma and carcinoma of the breast and kidney, where malignant cells have been reported to co-express CD40 and CD154 [14–17]. Indeed, the presence of CD154 in melanoma correlates with a more aggressive phenotype and shorter tumor-free patient survival compared to melanomas lacking CD154 [14]. In line with these associations, sustained expression of CD154 in CD40-positive immortalized epithelial cells confers oncogenic properties *in vitro*, increasing their proliferation, motility and invasion [16,17]. The migration of human multiple myeloma cells has also been reported to increase following CD40 stimulation, an effect mediated *via* the PI3 kinase and NF-κB signalling pathways [18]. The chronic production of CD154 in the tumor microenvironment may have additional, indirect effects on tumor growth by facilitating angiogenesis through the activation of CD40 in endothelial cells, as recently shown in a mouse model of mammary carcinogenesis [19]. Moreover, CD40 engagement in neutrophils signals for oxidative burst and the release of reactive oxygen species and matrix metalloproteinases which may contribute to transformation and metastatic spread [20].

It is also of interest that many of the phenotypic effects of dysregulated CD40 signalling on B cells are mimicked by the expression of the viral oncogene latent membrane protein 1 (LMP1) of Epstein–Barr virus [21]. Similar to stimulated CD40, LMP1 interacts with TRAFs, activates the NF-κB and MAPK pathways and promotes the proliferation of normal B lymphocytes. However, whereas CD40 requires engagement by its ligand to execute these functions, LMP1 is constitutively active by virtue of its six transmembrane domains which enable ligand-independent aggregation of the cytoplasmic C-terminus of the protein and signal activation [22]. Elegant studies using a chimeric LMP:CD40 molecule comprising the transmembrane domains of LMP1 fused to the cytoplasmic tail of CD40 support the notion that constitutive engagement of the CD40 pathway might contribute to malignancy. Thus, expression of the LMP:CD40 chimaera was found to promote the oncogenic transformation of immortalized fibroblasts *in vitro* [17] and to induce lymphomas when targeted to the B cell compartment of transgenic mice [23].

3. The CD40 pathway as a key regulator of the anti-tumor immune response

At first glance, the aforementioned observations appear to point to a positive role for the CD40 pathway in the pathogenesis of cancer. This is at odds, however, with the increased frequency of lymphomas and carcinomas in hyper-IgM syndrome patients [24], indicating that the effects of CD154 on malignant growth are likely to be more complex than thought. In line with this notion, various mechanisms by which tumors deregulate the CD40 pathway to safeguard their growth have been reported. Thus, $CD4^+$ T cells from patients with chronic lymphocytic leukaemia (CLL) display impaired mobilization of CD154 upon activation *in vitro*, and the leukaemic cells suppress CD154 expression in co-cultured allogeneic T lymphocytes [25]. Modulation of CD154 expression by CLL cells may therefore provide a means to escape immune surveillance. Hock et al. reported that patients with hematologic malignancies such as acute myeloid leukaemia and multiple myeloma have increased levels of soluble CD40 receptor (sCD40) which reduces CD154 availability and correlates with poor patient prognosis [26]. Moreover, in a study of 1776 non-Hodgkin lymphoma patients and 2482 controls, enhanced risk of lymphomagenesis was found to associate with single nucleotide polymorphisms in the promoter and intronic sequences of the *CD40* gene that result in reduced expression of the receptor in dendritic cells [27]. These findings suggest that inhibition of the CD40 pathway may impede the immune system from mounting appropriate humoral and cell-mediated responses against precancerous cells.

Indeed, the CD40 pathway has been firmly recognized as a major component of the anti-tumor immune response. Tumor-specific immunity largely relies on professional antigen-presenting cells, mainly dendritic cells (DCs), which cross-present tumor antigens to T lymphocytes and prime them for activation. Effective priming of $CD8^+$ cytotoxic T lymphocytes (CTLs) depends on the maturation of the DCs, a process that is physiologically controlled by the

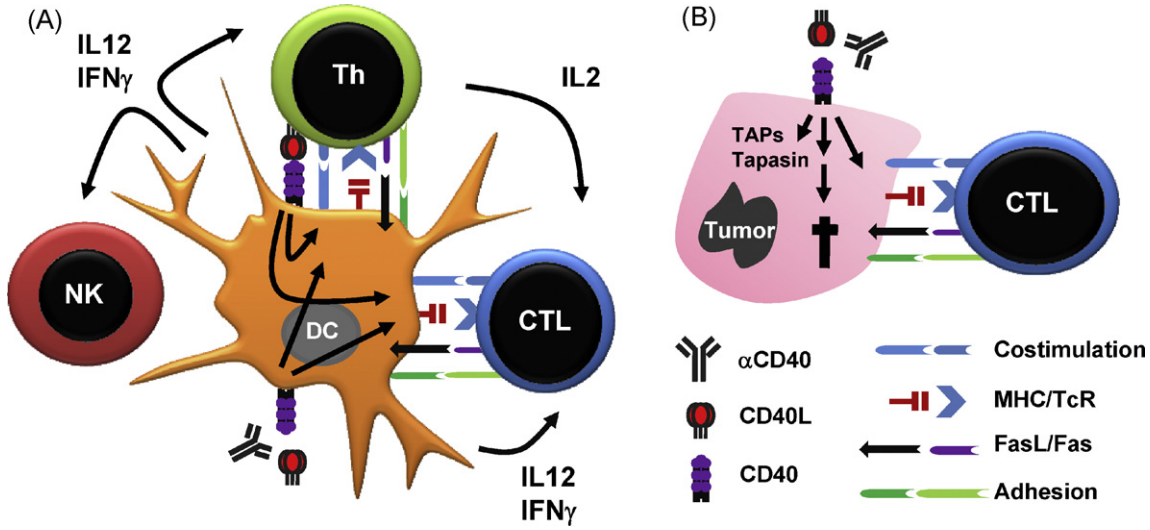

Fig. 1. The multiple anti-tumor properties of CD40 stimulation. (A) The engagement of CD40 on dendritic cells (DC) by CD154 expressed on $CD4^+$ T helper (Th) lymphocytes or, in a therapeutic setting, by CD40 agonists induces intracellular signalling pathways that result in expression of cytokines and co-stimulatory molecules important for $CD8^+$ cytotoxic T lymphocyte (CTL) priming and natural killer (NK) cell function. CTL and NK cells may then target tumor cells independently of their CD40 status (see text for details). (B) CD40-positive lymphoma or carcinoma cells respond to CD154 by up-regulating components of the antigen processing and presentation machinery such as MHC, TAPs and tapasin, as well as adhesion and co-stimulatory molecules which facilitate CTL recognition and killing. CD40 ligation may also induce direct anti-proliferative and pro-apoptotic effects on some tumor cells.

engagement of CD40 on DCs with CD154 expressed on antigen-stimulated $CD4^+$ T helper cells (Fig. 1A). CD40-stimulated DCs up-regulate antigen processing and presentation pathways and migrate to the lymph nodes to activate naïve T cells via MHC I/II and TcR engagement provided together with co-stimulation and cytokine signalling. In three seminal articles published in 1999, agonist CD40 antibodies were shown to substitute the function of $CD4^+$ lymphocytes in murine models of T-cell–mediated immunity (Fig. 1A), resulting in rapid expansion of CTLs that cleared established lymphomas and provided long-term protection against tumor rechallenge [28–30]. These observations provided an explanation for the impaired tumor antigen-specific CTL activation in CD40-deficient mice [31] and confirmed the key role of the CD40 pathway in the regulation of the anti-tumor immunity.

Collectively, the aforementioned observations raised the possibility of the potential exploitation of the CD40 pathway for cancer immunotherapy. Initial pre-clinical studies demonstrated that stable expression of the *CD154* gene in a small proportion of tumor cells was sufficient to stimulate a long-lasting systemic anti-tumor immune response that was dependent upon $CD8^+$ CTLs [32]. Other studies have used a recombinant adenovirus (RAd-CD154) to deliver CD154 either directly to tumors or to DCs in syngeneic models of chronic lymphocytic leukaemia, bladder, colorectal, prostate, lung carcinoma and melanoma. These treatments resulted in sustained tumor regression, even of weakly immunogenic or CD40-negative tumors [33–37]. The role of DCs in this effect was confirmed by infecting DCs *ex vivo* with RAd-CD154 and then injecting the modified cells into subcutaneous tumors. This approach not only eradicated the injected tumor nodule but also induced regression of untreated tumors in the same mouse and protected against subsequent tumor challenge [38]. More recently, an attenuated *Salmonella typhimurium* vaccine was utilized to deliver orally the CD154 transgene in a mouse model of B cell lymphoma. It was found that the modified bacterium induced significant protection against the development of lymphoma through the activation of DCs and the recruitment of Fas ligand-expressing tumor-infiltrating lymphocytes [39]. In addition to DCs, the activation of macrophages and natural killer (NK) cells appears to contribute to the *in vivo* anti-tumor effects of CD40 agonists [40,41], an observation that may partly explain why CD154 therapy is also efficacious in T cell deficient mice [42].

The therapeutic effects of CD154 also associate with a major reprogramming of the tumor microenvironment. Both transformed and adjacent host immune cells have been noted to produce a variety of soluble factors which favour malignant growth. Thus, the local immune response becomes polarized towards a pro-tumorigenic Th2 inflammatory function, typified by the influx of immature dendritic cells and regulatory T cells (Treg; $CD4^+/CD25^+/Foxp3^+$) that display a characteristic anergic phenotype and suppress the activity of cytotoxic T lymphocytes and natural killer cells. Interestingly, intratumoral administration of CD154 has been found to reduce the levels of the Th2 cytokines IL10 and TGFβ and to up-regulate the Th1 cytokines IL12 and IFNγ with concomitant depletion of Treg and increase in mature DCs [35,43,44]. In addition, CD40-stimulation on endothelial cells up-regulates VCAM-1, ICAM-1 and E-selectin which facilitates lymphocyte attachment, rolling and transmigration into the inflamed site as lymphocytes express LFA-1. Monocytes and macrophages expressing Mac-1 also bind endothelial ICAM-1 and transmigrate. As a result, CD40-mediated signalling may be crucial in promoting the migration of tumor-reactive immune effector cells into the tumor tissue [45,46] (Fig. 2).

CD40 agonists are therefore likely to have enormous potential in cancer immunotherapy by operating normal lymphoid cells to stimulate or correct the anti-tumor immune response, irrespective of the CD40 status of the cancer cells. However, the expression of CD40 in a variety of human malignancies indicates functional outcomes that might provide additional therapeutic opportunities.

4. The CD40 pathway directly enhances the immunogenicity of cancer cells

Cancer is typified by the outgrowth of poorly immunogenic cell clones which evade immune surveillance. Immunogenicity largely depends on a functional machinery that enables processing and presentation of intracellular tumor antigens by MHC class I to $CD8^+$ CTLs. This process is elegantly orchestrated by a large multicatalytic protease complex, the immunoproteasome, and the transporters associated with antigen processing TAP1 and TAP2 which convey peptides into the endoplasmic reticulum for complex formation with MHC class I molecules. The chaperone protein tapasin also contributes to this process by promoting the assembly of the MHC class I loading complex.

Components of this machinery are frequently impaired in cancer. For example, coordinated loss of TAP and MHC class I expression occurs in a number of human malignancies including Burkitt lymphoma and cervical and lung carcinomas and limits tumor cell recognition by $CD8^+$ CTLs [47–49]. Moreover, tapasin down-regulation associates with tumor progression in human melanoma [50]. Additionally, malignant cells display defects in the expression of co-stimulatory molecules which are important for CTL activation and function.

There is accumulating evidence to suggest that signalling via CD40 may re-activate the antigen-presenting functions of malignant cells and partly restore their *in vitro* recognition and killing by CTLs. Thus, CD40 ligation up-regulates the expression of cell surface markers and intracellular molecules associated with cell-to-cell contact and antigen processing and presentation, such as ICAM-1, MHC class I, TAPs and tapasin, and increases CTL-mediated lysis of Burkitt lymphoma and cervical carcinoma cells expressing TAP-dependent tumor antigens [48,51] (Fig. 1B). Stimulation of B-chronic lymphocytic leukaemia, follicular lymphoma and acute myelogenous leukaemia cells with CD154 also up-regulates ICAM-1 and the co-stimulatory molecules CD70, B7.1 and B7.2 and induces the expression of Fas, enabling leukaemic cell recognition and killing by FasL-expressing CTLs [52] (Fig. 1B).

A recent study has described a mechanism by which CD40 stimulation restores malignant cell immunogenicity and has implicated NF-κB as a master regulator of TAP, tapasin and immunoproteasome gene expression. Thus, the activation of NF-κB by CD154 was found to both stimulate the rapid *de novo* synthesis of the transcription factor IRF-1 and prime the promoters of immunoproteasome and antigen transport genes for IRF-1 mediated transactivation [53]. CD40 ligation therefore orchestrates a 'feed-forward' transcriptional program which ensures the coordinated and optimal induction of the expression of genes involved in antigen processing and presentation.

Signalling via CD40 may also influence immunostimulatory cytokine production in malignant cells. Thus, CD40 stimulation of Hodgkin's lymphoma and ovarian carcinoma cells increased the secretion of IL-8 and the T cell-activating cytokine IL-6. Other studies have shown that CD40 ligation of primary acute lymphoblastic leukaemia cells induces the production of macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC) which serve as potent chemoattractants for the transendothelial migration of CTLs [54].

Therefore, treatment of CD40-positive malignant cells with CD154 may elicit a direct anti-tumor effect by improving both the initial phase of the immune response (antigen recognition) and the effector stage (cytotoxicity and cytokine secretion) via adhesion and co-stimulatory molecule up-regulation, increased endogenous antigen presentation and cytokine synthesis.

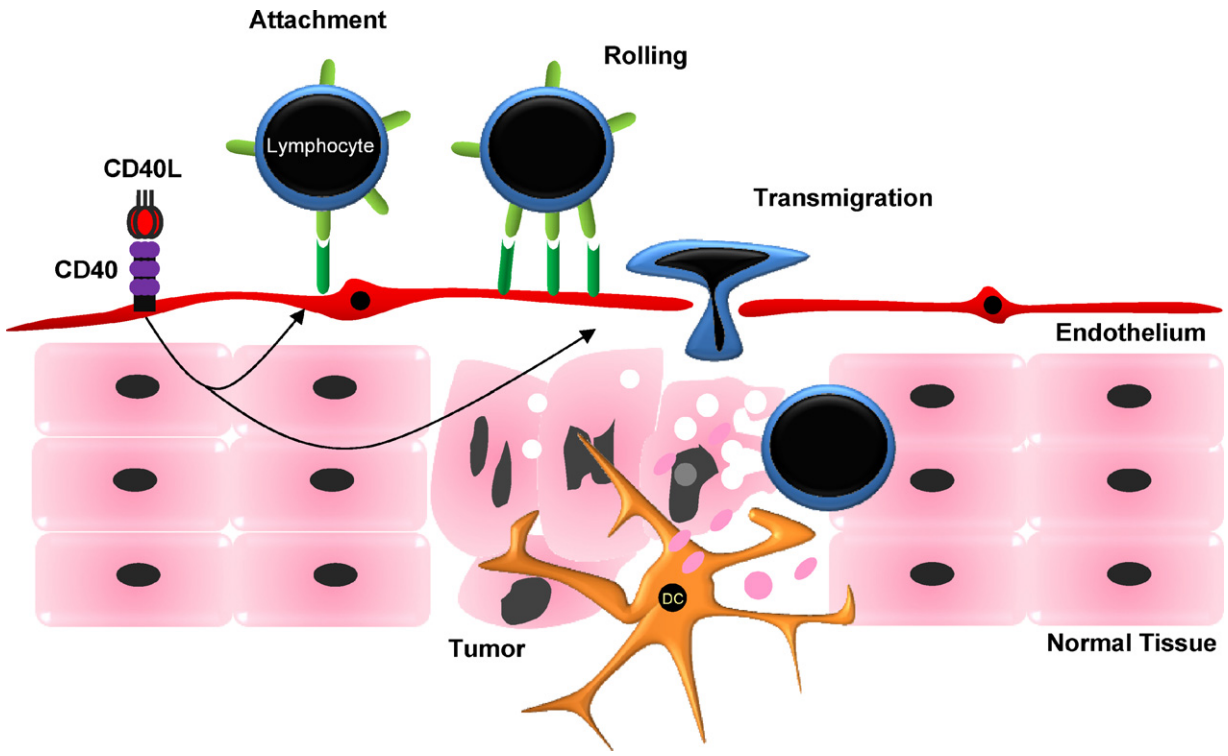

Fig. 2. CD40 signalling in endothelial cells may assist migration of tumor-reactive immune effector cells into the malignant tissue. The engagement of CD40 expressed on endothelial cells up-regulates VCAM-1, ICAM-1 and E-selectin which facilitate lymphocyte attachment, rolling and transmigration into the inflammatory tumor microenvironment. Monocytes and macrophages expressing Mac-1 also bind endothelial ICAM-1 and transmigrate. Both T lymphocytes and macrophages have been shown to mediate many of the anti-tumor properties of CD154.

5. Effects of CD40 ligation on malignant cell proliferation and survival

In addition to stimulating anti-tumor immune effects, CD40 engagement may directly influence malignant cell proliferation and survival. Thus, treatment of primary high-grade B cell lymphoma, multiple myeloma and Burkitt lymphoma cells with CD154 results in a marked decrease in proliferation both *in vitro* and in xenotransplanted mouse models [55–57]. Aggressive histology lymphomas also respond to CD40 ligation with sensitisation to apoptosis induced by chemotherapy, CD95/Fas engagement or serum withdrawal, an effect that has been attributed to up-regulation of pro-apoptotic gene products such as Bax and Fas, and the progressive loss of the survival protein cFLIP [58,59].

In marked contrast, CD40 ligation in low-grade B cell malignancies such as follicular lymphoma, chronic lymphocytic leukaemia and hairy cell leukaemia stimulates cell proliferation [60], indicating a differentiation state-dependent response to CD154. In these cells as well as in normal B lymphocytes, CD40 ligation protects from Fas-induced apoptosis by transcription-dependent and independent mechanisms. The former involves the activation of NF-κB which is required for the *de novo* synthesis of cellular inhibitors of apoptosis (cIAPs), cFLIP, Bcl-xL and Bfl-1/A1 [61,62] whereas the latter confers early rescue from Fas-mediated cytotoxicity through the stimulation of PI3-kinase/Akt survival signals downstream of TRAF6 [63]. Other studies have shown that whereas high (nanomolar) concentrations of recombinant CD154 suppress the proliferation of L3055 Burkitt lymphoma cells, low-level CD40 engagement by picomolar amounts of CD154 sustains growth in the same cell line [12]. Therefore, CD40–CD154 interactions may function more like a rheostat than an on/off switch, with CD154 operating in a graded manner to influence the outcome of CD40 stimulation.

The functional impact of CD40 ligation in solid tumors was addressed by early reports demonstrating that treatment of bladder, ovarian and skin carcinomas and melanomas with recombinant soluble CD154 results in inhibition of malignant cell proliferation *in vitro* [15,64,65] and in xenotransplanted mouse models [66,67]. Similar to the 'death receptors' of the TNF family, namely TNF receptor-1, Fas and TRAIL receptors, CD40 ligation stimulates the parallel induction of two competing pathways that influence carcinoma cell survival *versus* death. Thus, the pro-apoptotic effects of CD154 on ovarian, cervical, breast and bladder tumor cell lines are revealed upon disruption of the PI3 kinase/mTOR and ERK signalling pathways [68] or inhibitors of *de novo* protein synthesis [51,69]. Importantly, chemotherapeutic agents used in the oncological armamentarium, such as 5-fluorouracil, mitomycin C and etoposide, mimic protein synthesis inhibition and enhance the susceptibility of carcinoma cells to CD154-mediated killing [42,51]. The cytoplasmic tail of CD40 lacks a 'death domain' which typifies the cytotoxic receptors of the TNF family and the nature of the CD40-triggered apoptotic pathway is currently inconclusive. Some studies have implicated an autocrine/paracrine mechanism involving production of death ligands capable of inducing caspase-8 dependent carcinoma cell death [66,70–72] whereas a more recent report has shown that CD40-mediated apoptosis occurs via a TRAF3-JNK pathway that promotes caspase-9 activation [73]. Irrespective of the precise mechanism by which CD154 stimulates apoptosis in carcinoma cells, the finding that normal epithelial cells are spared of this cytotoxic effect [74] further highlights the therapeutic potential of the CD40 pathway in solid tumors.

6. CD40-directed therapies to treat human cancer

Various human malignancies have been treated with CD40-directed therapies in a total of six published clinical trials with

several others being in progress, according to the trial registration site at the National Institutes of Health (*clinicaltrials.gov*). The first published study was performed on patients with B-CLL. *Ex vivo* RAd-CD154-transduced autologous B-CLL cells were administered intravenously to 11 patients and were found to be well tolerated although the patients developed transient flu-like symptoms and less common side effects such as increased transaminase levels. Importantly however, this treatment led to a significant decrease in leukaemic cell count and lymph node size which associated with elevated number of total circulating T cells and $CD4^+$ and $CD8^+$ subsets [75]. In another trial, a subcutaneous vaccine consisting of *ex vivo* generated CLL tumors expressing CD154 and IL2 was evaluated in 9 B-CLL patients. Observed side effects included local pain, redness and swelling at the injection site. Three patients had more than 50% reduction of engaged lymph nodes within 12 weeks and 8 patients had stable disease after 6–23 months [76]. The same approach was applied in 10 patients with acute leukaemia (pre-B-ALL and AML), observing a 90% 5-year survival [77]. Tumor vaccines mixed with a bystander cell line expressing CD154 and GM-CSF were given intradermally to patients with stage IV disease (melanoma $n = 13$, renal cell carcinoma $n = 4$, mantel cell lymphoma $n = 4$, sarcoma $n = 3$, small cell lung cancer $n = 1$, lung cancer $n = 1$). No other toxicity than mild erythema and induration at the vaccine injection site was seen. The majority of patients had stable disease at 3 months follow-up, five of whom were still alive 2 years post-vaccination [78].

In a clinical trial published in 2001, recombinant soluble CD154 was administered subcutaneously to patients with advanced solid tumors or non-Hodgkin's lymphoma. Interestingly, one patient with a severe pharyngeal cancer showed complete response and remained disease-free for 2 years following entry into the trial and another patient had a partial response [79]. Phase I clinical trials using the agonist CD40 antibodies CP-870,893 and SGN-40 have also demonstrated anti-tumor efficacy particularly in melanoma and non-Hodgkin's lymphoma patients, respectively [80,81]. A most encouraging observation from these early studies has been the absence of major toxicity and other side effects, in light of understandable concerns regarding possible autoimmune or oncogenic activities mediated by CD40 activation. Although the clinical application of CD40 agonists has achieved objective anti-tumor responses, it is likely that the greatest potential for these agents will be in combination with conventional (e.g. 5FU) or novel modalities (e.g. oncolytic viruses) which display enhanced efficacy in pre-clinical animal models [42,82].

7. Conclusive remarks

The CD40 pathway holds promise as a novel therapy for advanced human malignancies. The therapeutic potential of CD40 agonists is highlighted by the multiple anti-tumor activities they could exert and include: (a) the stimulation of host antigen-presenting cells which orchestrate the induction of CTL and natural killer cell responses; (b) the activation of antigen-presenting functions in malignant cells; (c) the normalization of the tumor microenvironment and (d) the activation of direct anti-growth signals in CD40-positive tumors. The CD40 pathway therefore provides an opportunity to muster a variety of anti-cancer approaches in one therapy and, in combination with other modalities, offers an attractive option for clinical trials. Early clinical studies have confirmed that CD40 therapies are well tolerated and associate with anti-tumor activity. Exactly which patient cohorts could benefit most from CD40-directed therapies requires careful evaluation, particularly in light of the 'Janus' potential of the CD40 pathway to confer oncogenic properties when chronically engaged. A better understanding of the multifaceted biological properties of CD40 will undoubtedly encourage the design of novel and more effective therapeutic strategies for human cancer.

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