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REVIEW

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Renewed interest in cancer immunotherapy with the tumor necrosis factor superfamily molecules

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Abstract Molecules belonging to the Tumor Necrosis Factor (TNF) and TNF receptor superfamilies have explosively expanded through the era of genomics and bioinformatics. Biological investigations of these molecules have explored their potency as attractive targets for cancer therapy. Anti-tumor mechanisms mediated by TNF superfamily molecules (TNFSF) could be classified into direct actions onto tumor cells and indirect effects through immune or non-immune components of tumor-bearing host. In this review, we focus on TRAIL, CD40, 4-1BB (CD137), and LIGHT as promising molecules to mediate powerful and selective anti-tumor responses, and summarize their unique effector mechanisms. In addition, optimal approaches to manipulate these molecules for cancer therapy are also discussed. We try to provide an insight into a role of TNFSF in cancer therapeutics and highlight each of their potency to be an important player in anti-cancer strategies.

Introduction

Tumor necrosis factor (TNF) was originally characterized as a substance mediating necrotic death in various types of tumors in 1975 [4]. As a consequence of the findings, numerous challenges to utilize TNF for the treatment of cancer had been implemented in both experimental animals and clinical trials. Although certain levels of immunological and tumoricidal responses have been observed in experimental tumor models, clinical trials with systemic administration of recombinant human TNF were unsuccessful due to considerable adverse effects without apparent therapeutic benefits [30, 33]. Beginning in 1990s, novel molecules belonging to TNF and TNF receptor superfamily (referred to as TNFSF and TNFRSF hereafter) have been identified and characterized. Developments of world-wide organizations constructing genomic and expressed sequence tags (EST) databases of mammals as well as the emergence of bioinformatics have boosted the expansion of these families. Among novel members of TNFSF and TNFRSF, molecules that mediate powerful anti-tumor effects without inducing severe adverse effects have been detected. Thus, growing TNFSF and TNFRSF molecules have brought us the revival of TNF-related molecules in cancer immunotherapy. In this review, we will discuss the molecules of this superfamily as potential targets for the development of future cancer immunotherapy.

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Mechanistic insights of cancer immunotherapy with TNFSF/TNFRSF molecules

Cancer immunotherapies employing TNFSF/TNFRSF molecules exhibit anti-tumor effects through two predominant mechanisms: direct killing of tumor cells and indirect effects by activated anti-tumor immunity. The former mechanism is limited to tumors which express appropriate TNFRSF molecules, while the latter works irrelevant to tumor types so that it may have broad applicability as cancer therapy. These mechanisms are not mutually exclusive, and some TNFSF molecules employ both mechanisms to express anti-tumor effects.

Direct anti-tumor effects through TNFRSF molecules on tumor cells

Signaling from several TNFRSF molecules directly induces cellular phenotypic changes that result in death

of tumor cells. One representative mechanism is to deliver apoptotic signal from death domain-containing TNFRSF molecules on tumor cells. Practical application of this strategy to treat cancer, however, was severely hampered by profound adverse effects such as systemic inflammation and liver toxicity triggered by TNFα and Fas signaling, respectively [8, 9, 30, 31, 33, 56]. To overcome this, two alternative approaches have been developed. One is to discover TNFSF molecules capable of delivering death signals on tumor cells, without inducing significant damage in non-malignant cells. In this regard, TRAIL and CD40 are particularly attractive targets. The other strategy is a selective administration technique into tumor sites such as isolated limb perfusion of TNFα.

TRAIL, a new path to selective tumoricidal effects

Among TNFRSF molecules with death domain, a rare example to show selective killing of malignant cells is TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptors system. TRAIL is a type-II transmembrane protein expressed as a homotrimer [26, 43], shows high similarity with TNFα and Fas ligand, and is capable of binding to five different receptors [11, 15, 47, 48, 64]. Two of the receptors, death receptor 4 (DR4) and DR5, have cytoplasmic death domains so as to deliver apoptotic signals [47, 48, 64]. The other three receptors are decoy receptor 1 (DcR1), DcR2, and osteoprotegerin (OPG), which are either devoid of functional death domains or produced as secreted protein, and therefore may act as a negative regulator of cell death [11, 15, 47, 64]. TRAIL expression can be induced on activated T, NK, dendritic cells (DC), and monocytes, while a subset of liver NK cells constitutively express [68]. Importantly, many tumor cells are susceptible to TRAIL-induced apoptosis, whereas non-transformed cells are in general resistant to TRAIL. Although the precise mechanisms underlying the differential sensitivity remain unclear, several possibilities have been proposed. First, an obvious hypothesis would suggest a role of functional balance between death domain-containing receptors and decoy receptors. Large-scale screening of different cell lines, however, does not always indicate the correlation between TRAIL sensitivity and receptor expression pattern [34, 85]. Alternative possibility is a distinct transduction of death signal between tumor and normal cells. The primary candidate responsible for the distinct signaling is cellular FLICE-like inhibitory protein (c-FLIP), which prevents apoptosis by blocking caspase-8 activation [28]. Increased expression of c-FLIP in TRAIL-resistant cell lines and a gain of susceptibility by decreasing c-FLIP expression in those lines have been reported [21, 34].

There are numerous studies indicating potential application of TRAIL for cancer immunotherapy. Recombinant TRAIL or anti-DR5 mAb has demonstrated remarkable anti-tumor effects that eradicate established tumors in experimental animals with no or very little adverse effects [27, 75]. The mice genetically deficient of the TRAIL gene exhibit increased susceptibility to experimental and spontaneous tumor [10], suggesting an important role of endogenous TRAIL in tumor surveillance. This notion was further supported by the findings that fibrosarcoma cells grown in the mice treated with anti-TRAIL neutralizing mAb, have increased susceptibility to TRAIL-induced death [69]. Interestingly, TRAIL-deficient T cells exhibit significantly lower activity of graft-versus-leukemia (GVL) effects compared to wild-type T cells, whereas these T cells generate comparable graft-versus-host disease (GVHD) [59]. This study may suggest a potential use of TRAIL to strengthen GVL effects without exacerbating GVHD. In addition, combined usage of chemotherapeutic drugs with TRAIL sensitizes tumor cells otherwise resistant to TRAIL-induced death [24]. Accumulated pre-clinical studies thus clearly indicate a potential of TRAIL for cancer therapy.

Tumoricidal effects of CD40 signaling on tumor cells

Another example for the direct tumoricidal effect through TNFRSF molecules on tumor cells is CD40 and CD40L (CD154) system. CD40 is widely expressed on various types of cancer including hematological malignancies and epithelial cell-derived carcinomas. In hematopoietic tumors, signaling from CD40 leads to diverse outcomes according to cell types. In Hodgkin's disease and low-grade B cell malignancies such as chronic lymphocytic leukemia, hairy cell leukemia, and follicular lymphoma, CD40 activation contributes to the survival of tumor cells through increased proliferation and inhibited apoptosis [16, 32, 74], as similar to its effects on non-malignant B cells. In contrast, CD40 signaling in high-grade B cell lymphoma, Burkitt lymphoma, and multiple myeloma cells induces growth arrest and apoptosis [17, 50, 57]. In epithelial carcinoma cells, the effects of CD40 signaling appear consistently suppressive, as it mediates growth retardation and apoptosis in breast, ovarian, squamous cell, and lung cancer [19, 25, 52, 86].

Although in vitro studies indicate the direct tumoricidal activity of CD40, in vivo anti-tumor effects could be interpreted as indirect actions to tumor cells because of its broad expression and functions on immune cells [53]. In this regard, recombinant CD154 protein is capable of inhibiting the growth of $CD40^{+}$ human breast or ovarian tumor xenografted in severe combined immunodeficiency (SCID) mice [19, 25]. These results suggest that in vivo anti-tumor effects by CD40 signaling are, at least in part, mediated independently of adaptive immune systems. Increased expression of apoptotic molecules such as Fas ligand, TNFα, and TRAIL would contribute to the direct tumoricidal effects of CD40 [1, 14, 20]. Alternatively, CD40 signal in B cell lymphoma converts them to suitable antigen-presenting cells (APC)

by increasing costimulatory molecule and/or cytokine expressions [61]. This mechanism has been translated into clinic, in which autologous plasma cell leukemia are stimulated with CD154 ex vivo and used as cancer vaccine [62].

Isolated limb perfusion of TNFα

Due to the dose-limiting toxicity, a tolerable dose of systemic TNFα is 10–50 times lower than that required for anti-tumor effects [2]. Isolated limb perfusion, a technique to achieve an elevated concentration of drugs at the isolated extremity without flowing them into systemic circulation, has been successfully applied to local administration of TNFα. Clinical trials by multiple groups have demonstrated that isolated limb perfusion of TNFα with melphalan achieves >70% response rate (complete and partial responses) in patients suffering from unresectable bulk melanoma or soft-tissue sarcomas [13, 35]. This approach thus can avoid the necessity of amputation of limbs. Therapeutic mechanism of isolated limb perfusion of TNFα appears to be destruction of endothelial cells and vasculature of tumors rather than direct killing of tumor cells [46, 54]. In addition, given the profound synergistic effects of TNFα and melphalan, augmented tissue penetration of chemotherapeutic drugs by TNFα would play an important role. Host immune cells may also contribute to the effects since pre-irradiated lymphopenic animals are not susceptible to TNFα perfusion [37]. Taken together, TNFα can be a potent therapeutic reagent for tumors localized in extremity by utilizing the isolated limb perfusion technique.

Indirect anti-tumor effects of TNFRSF expressed on immune cells

Many members of TNFRSF have been shown to function as costimulatory molecules on T lymphocytes [77]. In addition, function and survival of DC can be regulated by signals from TNFRSF molecules [44, 55, 60, 79, 84]. Thus, two major components for T cell immunity, i.e. T cells and APC, are both controlled by TNFSF/TNFRSF interactions. Targeting these pathways, therefore, is a potent strategy for cancer immunotherapy. Here we focus on two representative molecules, 4-1BB (CD137) and LIGHT, based on their capacity to stimulate both T cell and DC, and to generate powerful anti-tumor immunity.

Potent anti-tumor immunity induced by 4-1BB signaling

There is ample evidence demonstrating that triggering 4-1BB signaling elicits robust anti-tumor immune responses in vivo [42, 77]. This effect is largely interpreted by 4-1BB signaling on tumor-specific T cells enhancing proliferation and CTL activity, and preventing activation-induced cell death [51, 65, 67]. Recent studies, however, have revealed diverse expression and functions of 4-1BB on immune cells, and have suggested novel mechanisms of its anti-tumor effects (Fig. 1).

First, 4-1BB signaling is able to prevent and rescue T cells from immune tolerance [81, 83]. In several animal models that render Ag-specific T cells anergic through tolerogenic Ag immunization, agonistic anti-4-1BB mAb abrogates T cell anergy induction and recovers responsiveness of those T cells [83]. Since there is substantial evidence indicating that tumor-reactive T cells are rendered functionally tolerant in tumor-bearing mice [49], the functional role of 4-1BB signaling in T cell anergy could be crucial to mediate anti-tumor effects. In addition, 4-1BB signal delivery, in conjunction with tumor Ag vaccination, breaks T cell ignorance to poorly immunogenic tumors, leading to an eradication of those tumors [81]. Besides T cell anergy and ignorance, recent studies suggest that 4-1BB signal has a functional role in $CD4^+CD25^+$ regulatory T cells, a central player maintaining T cell tolerance and immune homeostasis. 4-1BB is expressed on activated regulatory T cells, and stimulation of regulatory T cells by agonistic 4-1BB mAb abrogates their suppressive function [6]. Taken together, interference with T cell tolerance mechanisms by 4-1BB may play an important role in the anti-tumor effects.

Secondly, 4-1BB expression on non-T cell population including NK cells and DC has been explored, and functional contribution of this pathway to the immune activation is strongly implicated [18, 41, 79, 82]. In vivo depletion of NK cells abrogates anti-tumor effects of anti-4-1BB mAb in some tumor models [41]. 4-1BB signal stimulates in vitro proliferation and cytokine production of NK cells purified from RAG-deficient

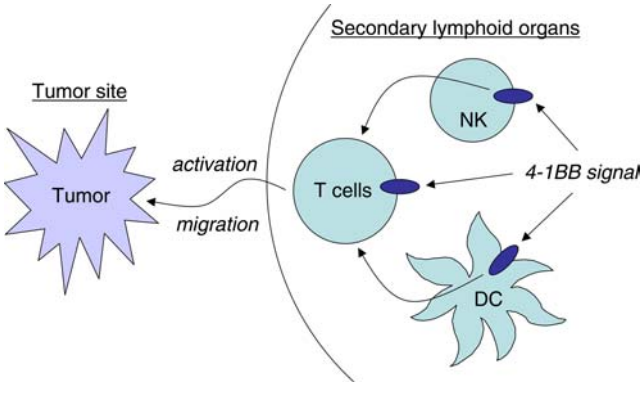

Fig. 1 Multi-cellular mechanisms of anti-tumor effects through *4-1BB signal*. During generation of anti-tumor immunity, 4-1BB signaling is capable of targeting at least three immune cell components, *T cells*, dendritic cells (*DC*), and *NK* cells. *4-1BB* signal directly activates tumor-specific T cells, while 4-1BB signal to *DC* and *NK* cells indirectly stimulate T cells through cytokines, cognate interaction, or other unknown mechanisms. T cell activation induced by 4-1BB signal confer them the ability to overcome T cell tolerance associated with tumor-bearing conditions, thus leaving secondary lymphoid organs to migrate into the tumor site and attack tumor cells

mice [82]. The NK cells activated with 4-1BB have a positive cross-talk with T cells, in which NK cells accelerate T cell responses to specific Ag and, on the other hand, T cell-derived IL-2 stimulates proliferation of NK cells [82]. 4-1BB expressed on DC may also function as immune modulator since stimulation of DC with 4-1BB ligand triggers IL-12 and IL-6 production and confers them potent Ag-presenting capacity [79]. Collectively, current studies strongly suggest that 4-1BB signaling activate the cross-talk between innate and adaptive immune systems, by which powerful anti-tumor effects are generated.

Finally, 4-1BB stimulation modifies the distribution pattern of tumor-specific T cells in vivo. The number of tumor-specific T cells infiltrating into the tumor sites significantly increases by the administration of agonistic anti-4-1BB mAb [80]. This effect is largely dependent on IFN-γ, since the infiltration of tumor-specific T cells is completely abrogated in mice deficient of IFN-γ, while their number in tumor-draining LN remains unchanged. Consequently, 4-1BB mAb is incapable of inducing anti-tumor effects in the mice deficient of IFN-γ or treated with anti-IFN-γ neutralizing mAb [80]. In addition, blockade of 4-1BB signaling by either anti-4-1BB ligand mAb or gene disruption of 4-1BB, decreases T cell infiltration in cardiac allograft and prolongs the graft survival [5]. Taken together, modification of migratory features in T cells by 4-1BB signaling could be a novel mechanism contributing to the anti-tumor effects.

Dual functions of LIGHT on tumor immunity through two counter-receptors

LIGHT is a potent T cell costimulator in both mouse and human immune systems [70, 71]. It is expressed on activated T cells and immature DC and interacts with two distinct cell-membrane receptors, HVEM and LTβR, and one decoy receptor, TR6/DcR3 [38, 70, 89]. HVEM is expressed on a broad range of hematopoietic cells including T cells, whereas LTβR is mainly detected on non-hematopoietic populations such as stromal cells [3, 23], suggesting a primary role of HVEM in T cell costimulation by LIGHT. In addition, it was also reported that HVEM signaling on DC stimulates their APC function and cytokine production [44]. Genetic disruption of LIGHT results in deficient CD8 T cell functions including impaired graft rejection in allogeneic organ transplantation [58, 72, 87]. Conversely, transgenic expression of LIGHT under T cell-specific promoters results in autoimmune phenotypes associated with the infiltration of activated T cells in multiple organs [63, 76]. These findings indicate that LIGHT–HVEM pathway plays an important role in the competent activation of T cell immunity as well as maintenance of T cell tolerance.

Consistent with its costimulatory functions, expression of LIGHT on tumor cells accelerates anti-tumor T cell immunity, which results in a delayed growth or spontaneous regression of tumors [71, 90]. Increased CTL activity in tumor-draining LN and abrogation of anti-tumor effect by $CD8^+$ T cell depletion, indicates a central role of tumor-reactive CTL in LIGHT-mediated anti-tumor effects. It was shown that inoculation of LIGHT-expressing tumor cells induces profound expression of chemokine CCL21 and adhesion molecule MAdCAM-1 on tumor stromal cells through LIGHT–LTβR interaction [90]. These factors may attract naïve T cells into the site of tumor, where they receive costimulatory signal by LIGHT–HVEM pathway to proliferate and differentiate into effector T cells against tumor (Fig. 2). Although the direct link between this phenomenon and anti-tumor effects by LIGHT has not been established, LIGHT-dependent manipulation of two distinct arms of immunity, i.e. migration and activation of T cells, is an attractive and novel strategy for cancer immunotherapy.

Approaches to manipulate TNFSF/TNFRSF molecules for cancer therapy

Gene transfer of costimulatory TNFSF molecules into tumor cells is a powerful cancer vaccine strategy in experimental models [7, 40, 71]. However, this method may encompass obstacles to translate into clinical settings due to requirements of gene transfer into primary tumor cells. Alternatively, recombinant proteins of TNFSF molecules can be prepared in vitro and used as biological adjuvants to enhance T cell responses triggered by cancer vaccines. Based on physiological structure of TNFSF proteins as homo- or heterotrimers [36], protein engineering to trimerize recombinant proteins would be essential for the effects. In fact, conjugation of leucine zipper motif to TNFSF proteins accelerates to

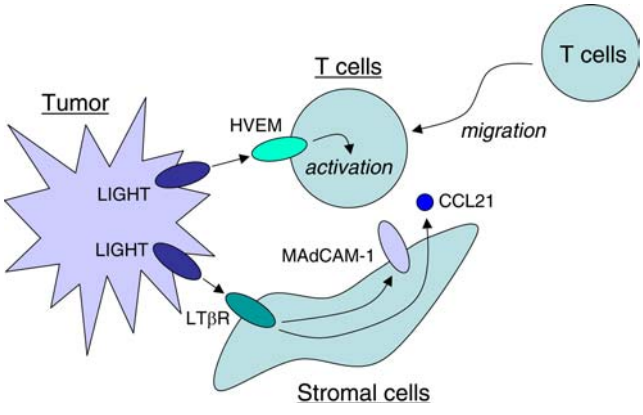

Fig. 2 Dual functions of *LIGHT* for the activation of anti-tumor immunity. LIGHT over-expressed on tumor cells triggers *LTβR* signal in tumor stromal cells to stimulate chemokine production and adhesion molecule expression such as *CCL21* and *MAdCAM-1*. These factors attract T cells into the tumor site where they receive *LIGHT–HVEM* costimulatory signal to be activated into anti-tumor effector T cells

form multi-complexes of proteins and results in superior biological effects in vitro and in vivo [22, 45, 75].

Development of agonistic mAb against TNFRSF molecules is one of the most promising strategies for cancer immunotherapy. Administration of agonistic mAb to 4-1BB, CD40, or OX-40 has been shown to generate strong anti-tumor responses in various experimental cancer models [12, 42, 66, 78]. To translate this strategy into clinical settings and to maximize their effects in patients, humanization of mAbs might be necessary. An alternative strategy to employ agonistic mAb for cancer immunotherapy is to construct membrane-bound single-chain Fv fragment (scFv). Recent study indicates that vaccination of anti-4-1BB scFv-expressing tumor cells is capable of treating MHC class I-negative parental tumor in a manner dependent on $CD4^+$ T cells and NK cells, but not $CD8^+$ T cells [88]. These findings suggest a potential use of scFv agonistic to costimulatory receptors to treat low immunogenic or immune-evaded human tumors.

Finally, immunotherapy with TNFSF/TNFRSF molecules can be significantly fortified by a combination with other vaccine strategies. For instance, the effects of agonistic 4-1BB mAb become prominent by concurrent vaccination with tumor-specific Ag or Ag-pulsed DC [29, 73, 81]. In adoptive immunotherapy using tumor-reactive T cells, ex vivo provision of 4-1BB signal induces continuous expansion of T cells and efficient anti-tumor activity after in vivo transfer [39]. In addition, tumoricidal activity of TRAIL has synergistic effects with various chemotherapeutic reagents [24]. Thus, identifying optimal combinations of TNFSF/TNFRSF-based treatments with other anti-cancer therapies would indeed be an important subject in future studies.

Concluding remarks

Wealthy knowledge in molecular nature and immunological functions of the TNF superfamily are now available and ready to be translated into the clinical settings for cancer therapeutics. Manipulation of TNFSF or TNFRSF molecules is an attractive strategy because of their pleiotropic functions on systemic cellular components including T cells, APC, non-hematopoietic cells such as stromal cells, or tumor cells themselves. Their effects include stimulation of anti-tumor immune cells, induction of cytokine and chemokine production, prolonged survival of effector cells, and direct lysis of tumor cells. These functions, if carefully selected and manipulated, represent new and promising strategies for cancer therapy.

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