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Review

Biochemical and Immunological Characteristics of 4-1BB (CD137) Receptor and Ligand and Potential Applications in Cancer Therapy

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Abstract. 4-1BB (CD137) is a member of the tumor necrosis factor receptor (TNFR) superfamily that is expressed primarily on activated T cells. Crosslinking of the 4-1BB receptor activates an intracellular signal cascade that leads to the activation of NF-κB and costimulation of T cell growth. Recent evidence indicates that 4-1BB may preferentially costimulate $CD8^+$ T cell growth and induce cytolytic activity. The cytolytic activity induced by 4-1BB crosslinking is able to eradicate large, well-established, poorly immunogenic tumors and augments allogenic T cell responses *in vivo*. The 4-1BB/4-1BB ligand costimulatory pathway can provide an alternative T cell costimulatory pathway in the absence of CD28, but may physiologically function as a synergistic or complementary pathway to the CD28 costimulatory pathway. Only some of the basic immunological functions of 4-1BB/4--1BB ligand have been elucidated and much study is required to determine its exact role in T cell activation.

Key words: tumor necrosis factor receptor; tumor; costimulation; T cell activation.

The molecules of the tumor necrosis factor receptor (TNFR) superfamily belong to type I glycoproteins (N terminus extracellular) and are comprised of at least 13 molecules, including Fas (CD95), CD30, TNFR I/II, OX40, CD40, CD27, HVEM/TR2, RANK and OPG. The family can be further divided into two subgroups with molecules such as 4-1BB, OX40, CD27, which are expressed mainly in lymphoid organs, and those molecules that have a wider tissue distribution, such as NGF-R, Fas, and TNFR I/II. 4-1BB (CD137/ILA) contains 4 extracellular cysteine-rich modules that have up to 30% homology to the other members of the TNFR family. The intracellular domain of 4-1BB has little homology to other TNFR family molecules except CD27[13].

Expression Patterns of 4-1BB and Its Ligand

The mouse 4-1BB gene was originally cloned by a modified differential screening procedure from concavalin A stimulated cytolytic T lymphocyte and helper T lymphocyte clone cDNA libraries[20]. 4-1BB is a 30 kDa type I membrane glycoprotein with 4 cysteine-rich extracellular modules and a hinge region near the transmembrane region rich in serine, threonine, and proline. It is expressed on the surface as a 55 kDa homodimer[33]. The human and murine forms of 4-1BB are 60% identical at the amino acid level[1]. Two different alternatively spliced transcripts of 4-1BB mRNA encode two different soluble forms of 4-1BB[28, 37]. The soluble forms of 4-1BB are detected in the supernatans of PHA or phorbol myristate acetate (PMA)/calcium ionophore stimulated peripheral blood mononuclear cells, but not in those of resting cells. Soluble 4-1BB can be detected at higher levels in the sera of rheumatoid arthritis patients compared to the sera from healthy donors, who have low or non-detectable levels of so-

luble 4-1BB[28]. Although the physiologic role of soluble 4-1BB in inflammatory states such as rheumatoid arthritis is not clear, one can hypothesize that soluble 4-1BB may somehow down-regulate responses to 4-1BB ligand by acting as a decoy receptor.

Surface expression of 4-1BB is detected on activated $CD8^+$ and $CD4^+$ T cells, activated thymocytes, intraepithelial lymphocytes, activated NK cells and eosinophils[12, 26, 30, 39]. Human 4-1BB mRNA is detectable in activated T cells, B cells, monocytes, and some IL-1β stimulated nonlymphoid cells[23, 35]. The surface expression of 4-1BB on monocytes and nonlymphoid cells has yet to be confirmed. However, 4-1BB was not detected on the surface of B cells[10]. After activation of T cells or T cell hybridomas by anti-CD3 monoclonal antibodies (mAb) or PMA/calcium ionophore, the expression of 4-1BB is up-regulated[7, 10, 31]. Both the human and mouse homologs of 4-1BB are expressed after T cell activation, though, human 4-1BB appears earlier after T cell activation than its mouse counterpart. Expression of 4-1BB on the cell surface peaks between 1–2 days and 6 days after activation for the human and mouse 4-1BB homologs, respectively[10, 16, 31]; the reason for this difference is not known. Murine NK cells also express high levels of 4-1BB, about one week after activation with IL-2, but it is not detectable in resting NK cells[26].

In two early studies, a variety of extracellular matrix proteins, including laminin and vitronectin, were thought to be the ligands for 4-1BB[3, 23]. Although the murine 4-1BB can bind extracellular matrix proteins, human 4-1BB does not. The functional significance of this binding is not clear. By an expression cloning approach, a high affinity ligand for 4-1BB was identified. 4-1BB ligand (4-1BBL) is a type II glycoprotein (C terminus extracellular) homologous to other members in the TNF ligand family including TNF, lymphotoxin α and β, CD40 ligand, and CD27 ligand[1, 12]. 4-1BBL is a 34 kDa glycoprotein expressed on the surface of activated T cells, macrophages, monocytes, dendritic cells, B cells, and B lymphomas[12]. The human and murine 4-1BBL have 36% amino acid identity. 4-1BBL can be up-regulated on the surface of some B lymphomas and purified B cells after exposure to cAMP or LPS, or stimulation through CD40 ligation[1, 7, 12, 15, 32]. Expression of 4-1BBL on T cells peaks 4 h after anti-CD3 mAb stimulation and decreases to background levels at 16 h and is undetectable on resting T cells[1].

Costimulation of T Cells by 4-1BB

It is well accepted that the T cell requires two signals for activation. Signal one is provided by the MHC peptide complex interaction with the T cell receptor, and signal two is provided by costimulatory molecules, with CD28 and B7 interaction being recognized as the key costimulatory signal. Recent studies indicate that 4-1BB is able to provide a potent costimulatory signal. Stimulation through 4-1BB in the presence of suboptimal doses of anti-CD3 mAb (signal one) causes T cells to proliferate and increase IL-2 production[1, 31, 36, 38]. In the absence of anti-CD3 mAb, stimulation through 4-1BB does not affect T cell function, indicating the 4-1BB is a costimulatory molecule. Initial experiments showed that disrupting 4-1BB/4-1BBL interaction with a soluble form of 4-1BB inhibits T cell growth. Similarly, a soluble form of 4-1BB inhibited a mixed lymphocyte reaction between BALB/c and C57BL/6 or C3H splenocytes[7, 8, 15]. However, soluble 4-1BB is unable to inhibit T cell proliferation if allogenic spleen cells are previously activated by CD40 cross-linking[8]. Blocking of T cell response to allogeneic antigens with soluble 4-1BB and soluble CTLA-4 (second counterreceptor for B7) showed an additive effect when compared to either soluble 4-1BB or soluble CTLA-4 alone[15]. These results suggest that both 4-1BB and CD28 pathways act independently to costimulate T cell growth.

4-1BB, as well as other members of the TNFR family, are emerging as important stimulatory molecules and markers for specific subsets of T cells. For example, OX40 is preferentially expressed on $CD4^+$ T cells[11, 24], and CD30 is expressed on Th2 cells[9, 29]. Similarly, 4-1BB preferentially activates $CD8^+$ T cells compared to $CD4^+$ T cells, with splenic $CD8^+$ T cells responding up to 25 times better to anti-CD3/anti-4--1BB stimulation than $CD4^+$ T cells *in vitro*[38]. Although both $CD4^+$ and $CD8^+$ T cells respond to anti-4--1BB mAb treatment in the context of anti-CD3 stimulation with increased proliferation when compared to anti-CD3 alone, $CD8^+$ T cells proliferate to a greater extent in response to anti-CD3 plus anti-4-1BB stimulation than anti-CD3 plus anti-CD28 stimulation. $CD4^+$ T cell proliferation was either the same or greater with anti-CD3 plus anti-CD28 mAb vs. anti-CD3 plus anti--4-1BB mAb[38]. As well as proliferating to a greater extent when treated with anti-4-1BB mAb *in vitro*, *in vivo* experiments in which tumor-bearing mice or mice undergoing acute graft-versus-host disease were treated with anti-4-1BB mAb displayed increased proliferative capacity and increased cytolytic activity[27, 38]. The preferential activation by anti-4-1BB mAb of $CD8^+$ T cells suggests that 4-1BB may have a large impact in modulating immune responses to intracellular pathogens, tumors, and also in the modulation of certain inflammatory states.

Several studies indicate that 4-1BB can provide a costimulatory signal to T cells in the absence of CD28 signaling. DeBennedette et. al.[7] showed that B lymphomas that do not express B7-1 and B7-2, but high levels of 4-1BBL can induce T cells to produce IL-2. T cells from $CD28^{-/-}$ mice are able to produce IL-2 in response to immobilized anti-CD3 and 4-1BB ligand or in response B cell lymphomas that express high levels of 4-1BBL but low B7-1/B7-2[6, 8, 34]. Although 4-1BB can send costimulatory signals in the absence of CD28 signals, the presence of both signals seems to synergize, and in the presence of 4-1BB the amount of the CD28 signal required for T cell stimulation is lower[8, 18]. The synergistic effect of 4-1BB and the CD28 signal is also demonstrated by the enhanced activity of cytolytic T lymphocytes and antitumor response to a poorly immunogenic sarcoma, AG104A[25]. Although 4-1BB can compensate for loss of the CD28 signal, in physiologic conditions the CD28 signal may be required to maximize the expression of 4-1BB on T cells. The requirement of the CD28 signal for 4-1BB expression is supported by the observation that cytolytic T lymphocyte clones had decreased 4-1BB expression and decreased cytolytic activity when restimulated in the presence of CTLA4Ig or a combination of anti-B7-1/anti-B7-2 mAb[25]. The presence of the CD28 signal may be important in the early phase of T cell activation, while 4-1BB may have a major role in clonal expansion of activated T cells. This is consistent with the finding that CD28 expression on T cells is rapidly down-regulated after T cell activation; in contrast, 4-1BB is up-regulated rapidly after B7-CD28 engagement.

The role of 4-1BB costimulation in the polarization of type I vs. type II cytokine production is not clear. T cells from CD28 knockout mice cultured with a B lymphoma expressing high levels of 4-1BBL produce both type I (IFN-γ) and type II cytokines (IL-4). Production of these cytokines is blocked by a soluble form of 4-1BB[6]. The natural ligand for 4-1BB may not induce as strong a signal as the antibody mediated ligation and, therefore, the cytokine responses mediated by 4-1BBL are not pronounced. Currently, there is no clear evidence indicating that the 4-1BB pathway is involved in differential cytokine expression.

Interaction of 4-1BB and 4-1BBL could initiate a bidirectional signal. When B cells are incubated with anti-μ chain antibodies and soluble 4-1BB, there is a synergistic increase in B cell proliferation[32]. Engagement of 4-1BBL can also activate monocytes to produce IL-6, IL-8, TNF-α, and inhibit IL-10 production[22], suggesting a role of 4-1BB in the inflammatory response.

Intracellular Signaling Pathways of 4-1BB

Although no intrinsic enzymatic activities have yet been assigned to the intracellular domain of 4-1BB, this domain contains a Cys-X-Cys-Pro motif that mediates binding to the src family kinase $p56^{lck}$. The $p56^{lck}$ interacts with the cytoplasmic tails of CD4 and CD8 and functions in TCR co-receptor signaling[4]. The significance of the interaction of the cytoplasmic domain of 4-1BB and $p56^{lck}$, however, is still unknown since 4-1BB is not phosphorylated, nor has another substrate for 4-1BB associated $p56^{lck}$ been detected[19].

It has been reported that the 4-1BB cytoplasmic domain interacts with the TNF receptor associated factor (TRAF), which is a family of proteins consisting of at least 6 members, TRAFs 1–6. The TRAFs are adaptor proteins that link many members of the TNFR family, including CD40, CD30, TNFR II and OX40, to downstream signaling pathways, such the JNK and NF-κB pathways, ultimately leading to T cell activation and production of IL-2. 4-1BB interacts with TRAFs 1 and 2, with 4-1BB having a higher affinity of interaction with TRAF 2[2]. There is only minimal association of 4-1BB with TRAFs 1 and 2 in resting T cells, but upon treatment with anti-4-1BB antibodies, there is increased TRAF 1 and 2 association with 4-1BB[34]. The TRAFs interact with one to two stretches of intracellular acidic residues of 4-1BB and this interaction leads to increased IL-2 production[17, 34]. The importance of TRAF association with 4-1BB in signal transduction is demonstrated by the lack of IL-2 production in response to activation of 4-1BB on T cells from TRAF 2 deficient mice or TRAF 2 dominant negative transgenic mice[34]. 4-1BB may, in addition to increasing IL-2 gene transcription and T cell activation, initiate a positive feedback loop to up-regulate its expression by virtue of the presence of binding sites for NF-κB in the promoter region of 4-1BB[21].

Immunotherapy of Cancer by Targeting the 4-1BB Costimulatory Pathway

4-1BB has been shown to be a potent costimulatory molecule. Previous studies with B7 have shown that provision of a strong costimulatory activity in *in vivo* tumor models can induce a potent antitumor response[5]. However, this antitumor response is ineffective for large established tumors and poorly immunogenic tumors[14]. 4-1BB is able to induce $CD4^+$ and $CD8^+$ T cell dependent antitumor responses against both poorly immunogenic tumors and established tumors as evidenced by the eradication of established immunogenic P815

mastocytoma and poorly immunogenic AG104A sarcoma with administration of agonistic anti-4-1BB antibodies. The antitumor response induced by anti-4-1BB antibodies was both curative and proved effective when cured mice were rechallenged with tumors, suggesting a long-lasting immunity was developed[27]. The 4-1BB stimulated antitumor response to P815 tumors is abrogated if NK cells are depleted with anti-asialo GM1 or anti-NK1.1 mAb[26]. Although NK cells are necessary for the induction of 4-1BB stimulated antitumor response, the antitumor response is not caused by direct NK cytolytic activity, as supported by the observation that there is outgrowth of P815 tumors in anti 4-1BB treated BALB/c nude mice[26]. Thus, NK cells may play a role in 4-1BB stimulated antitumor response. The specific role of the NK cell may be in the production of cytokines to promote the initial antitumor response and facilitate $CD8^+$ T cell activation. Anti-4-1BB agonistic antibodies were also effective in curing 50% of mice in a metastatic tumor model and proved to be more effective than similar treatment with anti-CD28 or anti-CTLA4 treatments[27].

To study whether the natural 4-1BB ligand receptor interaction can also induce an effective antitumor response, MELERO at al.[25] transduced 4-1BBL into P815 and AG104A. Mice that were inoculated with 4-1BBL transduced tumors remained tumor free and were able to resist rechallenge of the wild-type tumor, indicating the establishment of long term immunity to the tumors[25]. Transduction of 4-1BBL directly into tumor cells thus may allow direct priming of T cells and independence from the requirement for antigen presentation cells in the induction of a T cell response.

Conclusion

Clearly, 4-1BB holds a prominent place in the receptor ligand interactions that lead to T cell activation. Understanding the cellular and molecular events of the 4-1BB costimulatory pathway will advance our knowledge of receptor – ligand interactions in the generation of an immune response and provide insights into the design of novel approaches for the immunotherapy of cancer.

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