A BISPECIFIC MOLECULE TARGETING CD40 AND TUMOR ANTIGEN MESOTHELIN ENHANCES TUMOR-SPECIFIC IMMUNITY

Shiming Ye¹, Diane Cohen¹, Nicole A. Belmar¹, Donghee Choi¹, Siu Sze Tan¹, Mien Sho¹, Yoshiko Akamatsu¹, Han Kim¹, Ramesh Iyer², Jean Cabel³, Marc Lake², Danying Song², John Harlan², Catherine Zhang¹, Yuni Fang¹, Alan F. Wahl³, Patricia Culp³, Diane Hollenbaugh³, Debra T. Chao¹
1. AbbVie Biotherapeutics Inc., Redwood City, CA 94063
2. AbbVie Inc., 1 North Waukegan Rd., North Chicago, IL 60064
3. Former AbbVie Employee

Running title: CD40 activation by a tumor-targeted bispecific molecule

Keywords: CD40, Mesothelin, bispecific molecule, tumor targeting, immune therapy

*Corresponding Author
Shiming Ye, AbbVie Biotherapeutics Inc., 1500 Seaport Blvd, Redwood City, CA 94063.
Phone: 650-454-2746; Fax: 650-399-8746; E-mail: shiming.ye@abbvie.com

Disclosure of potential conflicts of interest
SY, DY, NAB, DC, SST, MS, YA, HK, RI, ML, DS, JH, CZ, YF and DTC are employees of AbbVie, and JC, AFW, PC and DH are former employees of AbbVie who were employed by AbbVie at the time of the study. SY, YA, AFW and PC are holders of pending patent (United States patent App. 15/606,200). The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.
ABSTRACT
Agonistic CD40 monoclonal antibodies (mAbs) have demonstrated some clinical activity, but with dose-limiting toxicity. To reduce systemic toxicity, we developed a bispecific molecule that was maximally active in the presence of a tumor antigen and had limited activity in the absence of the tumor antigen. LB-1 is a bispecific molecule containing single-chain Fv domains targeting mouse CD40 and the tumor antigen mesothelin. LB-1 exhibited enhanced activity upon binding to cell surface mesothelin but was less potent in the absence of mesothelin binding. In a mouse model implanted with syngeneic 4T1 tumors expressing cell surface mesothelin, LB-1 demonstrated comparable antitumor activity as an agonistic CD40 mAb but did not cause elevation of serum cytokines and liver enzymes as was observed in anti-CD40–treated mice. The results from our study of LB-1 were used to develop a human cross-reactive bispecific molecule (ABBV-428) that targeted human CD40 and mesothelin. ABBV-428 demonstrated enhanced activation of antigen-presenting cells and T cells upon binding to cell surface mesothelin, and inhibition of cultured or implanted PC3 tumor cell growth after immune activation. Although expression of cell surface mesothelin is necessary, the bispecific molecules induced immune-mediated antitumor activity against both mesothelin+ and mesothelin– tumor cells. ABBV-428 represents a class of bispecific molecules with conditional activity dependent on the binding of a tumor specific antigen, and such activity could potentially maximize antitumor potency while limiting systemic toxicity in clinical studies.

INTRODUCTION
CD40 is a key co-stimulatory molecule that functions as a master switch for both innate and adaptive immune systems [1-3]. An agonistic CD40 monoclonal antibody (mAb) can directly activate and "license" antigen-presenting cells (APCs) to prime effective cytotoxic T-cell responses with limited help from CD4+ T cells [4-6]. Agonist anti-CD40 has been explored in Phase 1 clinical trials [7-10]. Although they have shown efficacy, systemic dose-limiting toxicities, including elevated liver enzymes and cytokine release syndrome [7,8], have prevented CD40 agonists from achieving full antitumor potency in patients. To lower the systemic toxicity, intratumoral injection of agonistic anti-CD40 has been studied [11,12] and elicits antitumor response with limited systemic toxicity in preclinical studies [13-15]. However, due to the logistical complexities of intratumoral dosing in the clinical setting, it is desirable to engineer a tumor-targeted molecule that provides full antitumor potency but exhibits limited toxicity, thus enabling systemic dosing.

Tumor-targeted activation has been tested in various molecular platforms to reduce systemic toxicity [16-18]. For example, tumor targeted anti-CD3 bispecific molecules have been investigated in preclinical as well as in clinical studies [19,20]. A similar tumor-targeting strategy has also been used to generate IL2 immunocytokine to achieve tumor-localized activity of IL2 [21]. Probody is another tumor-targeted molecular platform that keeps the active domain masked by a peptide until it is cleaved within the tumor microenvironment by tumor-specific proteases [22]. A tumor-targeted CD40 molecule has been tested by conjugating a CD40 agonist mAb chemically with a tumor-homing peptide [23]. However, there are limited reports about recombinant bispecific molecules targeting both CD40 and tumor antigens to achieve maximal activity in the presence of the tumor-associated antigen (TAA) and minimal activity in its absence, therefore, inducing conditional CD40 activation. Such a bispecific molecule would limit CD40 activation in normal tissues with little or no TAA expression, thus reducing the likelihood of systemic toxicities as a result of CD40 activation. Mesothelin (MSLN) is a target that exhibits tumor-specific expression. MSLN is a GPI-anchored cell surface molecule and has low expression on a few normal tissues, such as mesothelial cells lining the pleura, peritoneum, and pericardium [24].
In contrast, MSLN has high expression on mesotheliomas and carcinomas of the pancreas, lung, and ovary [25]. Overexpression of MSLN in cancers can elicit humoral and cellular immune responses, likely due to the limited expression of this protein in normal tissues [26-28]. Therefore, targeting MSLN together with agonist anti-CD40 may help to enhance the immunogenicity of MSLN-expressing tumors by inducing active immune responses against the tumor rather than normal tissues.

LB-1 and ABBV-428 are bispecific molecules targeting mouse or human CD40 and MSLN with single-chain fragment variable (scFv) domain converted from variable chains of an agonistic anti-CD40 and anti-MSLN. The current study provides preclinical evidence demonstrating the conditional activity of this bispecific molecule and supports the development of a MSLN-targeted CD40 immune therapy to treat patients with MSLN+ cancers.

**METHODS**

**Cells and reagents**

HEK-293 (human embryonic kidney) (ATCC# CRL-1573), PC-3 (human prostate adenocarcinoma) (ATCC# CRL-1435), and mouse syngeneic tumor cell line 4T1 (mouse mammary carcinoma) (ATCC# CRL-2539) cells were purchased from ATCC and were stably transfected to express mouse or human CD40 or MSLN. The parental cell lines were authenticated by ATCC and were not authenticated in the past year. Human CD40 or MSLN gene was cloned into a CHEF1 expression vector [29]. 1 µg of vector DNA was transfected into 2.5X10^5 parental cells by FuGENE 6 transfection reagent according to the vendor’s instruction (Promega). The cells stably expressing CD40 or MSLN were selected in the medium containing G418 (0.5mg/mL, Thermo Fisher). All cell lines were tested negative for Mycoplasma and banked after purchasing or genetic modifications, and maintained in culture for no more than 10 passages from master cell banking. Fresh human peripheral blood mononuclear cells
(PBMCs) from healthy donors (about $10^9$ per donor) were purchased from AllCells. Donor virus testing results of HIV, HBV, and HCV were negative.

Antibody against human MSLN (clone K1) was purchased from Santa Cruz Biotech, CD86 (clone FUN-1) from BD Bioscience, and CD83 (clone HB15e) from eBioscience. The following anti-mouse antibodies were purchased from BD Bioscience: CD45 (clone 30-F11), B220 (clone RA3-6B2), CD19 (clone 1D3), CD86 (clone GL1), Ly6C (clone AL-21), Ly6G (clone 1A8), and CD11b (clone M1/70). The following molecules were produced at AbbVie [30]: agonist anti-mouse CD40 (1C10), agonist anti-human CD40, and anti-MSLN (human and murine cross-reactive), as well as tumor-targeted bispecific molecules LB-1 or ABBV-428 with specificities to murine or human CD40 and MSLN. All constructs were subcloned into pHyE vectors developed at AbbVie [31] and transiently transfected to 293-6E cells for production. The Fc function was monitored by a chromium-51($^{51}$Cr) release assay as described previously [32].

**Animals**

Six to eight week-old NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ (NSG) and BALB/cJ mice (The Jackson Laboratory) were housed under SPF conditions in an AAALAC accredited facility. Mice were acclimated for seven days prior to use and were kept on a 12/12-hour light/dark cycle with food and water given ad libitum. All animal procedures were reviewed and approved by the internal Institutional Animal Care and Use Committee (IACUC).

**Monitoring immune cell activation in vitro**

B cells were enriched using a column-free negative selection kit. Murine B cells were isolated using an EasySep mouse B cell enrichment kit (StemCell Technologies) from single-cell suspensions, which were prepared by pressing BALB/c mouse spleens through 100 µm nylon cell strainer (VWR) using the plunger end of a syringe in RPMI1640 with 10% FBS. Human B cells were isolated from human PBMCs using EasySep human B cell enrichment kits (StemCell Technologies) according to the vendor’s instruction. B cells were cultured in fully defined AIM V serum free culture
medium (Thermo Fisher Scientific). Human immature dendritic cells (DC) were derived from monocytes enriched from PBMCs by EasySep human monocyte enrichment kit (StemCell Technologies) and maintained in ultralow attachment polystyrene plates (Corning) in StemSep serum free medium (StemCell) supplemented with GM-CSF (10 ng/mL) and IL4 (20 ng/mL) (Peprotech) at 37°C, 5% CO₂ for 6 days. Purified B cells or immature DCs (5 x 10⁵/mL) were treated when cultured alone or cocultured with equal numbers of irradiated HEK293 cells or 4T1 cells with or without MSLN expression. In some experiments, recombinant human MSLN (Pepro Tech) were mixed in the cell culture at 300 nM. After 48 hours, cells were collected and stained for activation markers with fluorophore-conjugated antibodies (listed above). The staining was performed on ice for 15 minutes followed by washing for 3 times. The flow cytometry data were acquired using a LSR Fortessa (BD Biosciences) and analyzed using FlowJo (TreeStar Inc.). The supernatants of DC cultures (10 μl) were harvested and assayed for IL12p70 production by AlphaLISA (Perkin Elmer). B-cell proliferation was determined after treatment for 6 days by adding 0.5 μCi/well H³TdR (NEN) for the last 16 hours of culture and measuring H³TdR incorporation as counts per minute (CPM) by Wallac 1450 MicroBeta scintillation counter.

Human T cells (5 x 10⁴) were purified from PBMCs with EasySep human T cell enrichment kit (StemCell Technologies), cocultured with autologous monocyte-derived DCs (moDC) (5 x 10³) prepared as described above, and HEK293 cells (5 x 10³) with or without MSLN expression. T-cell activation was monitored by IFNγ production in the coculture under different treatments for 48 hours. IFNγ production was measured by ELISPOT assay. Briefly, cells were washed 4 times in Dulbecco’s phosphate-buffered saline (DPBS) and plated in an anti-IFNγ-coated PVDF 96-well plate for 24 hours (R&D Systems). IFNγ ELISPOT was developed per manufacturer’s instructions and was quantified by a C.T.L. Elispot plate reader (Cellular Technology Ltd) using ImmunoSpot Version 5.0 software.
In vitro tumor cell lysis assay

To set up the in vitro tumor lysis assay, the human prostate cancer cell line PC3 was transfected to express different amounts of human MSLN and co-express green fluorescence protein (GFP) by FuGENE 6 as described previously. In parallel, PC3 cells were also transduced with a NucLight Red™ Lentivirus reagent (Essen BioScience) to express red fluorescence protein (dsRed). MSLN expression on PC3 stable cells was evaluated by QIFIKIT (Agilent) according to the manufacture's instruction, and by IHC using anti-5B2 (ThermoFisher) on formalin-fixed paraffin embedded (FFPE) cell pellets after antigen retrieval and detection by DAB with secondary polymer kit (DAKO). Based on MSLN expression, the PC3 stable cells were defined as PC3-MSLN^hi, PC3-MSLN^med, and PC3-MSLN^low according to IHC score 4+, 3+ and 2+. PC3 cells expressing GFP or dsRed with differing expression of MSLN were plated at 5 × 10^3 cells per well in a 96 well plate for 24 hours prior to the addition of 5 × 10^3 moDCs and 5 × 10^4 T cells. The cells were then treated with antibodies or bispecific molecules (5µg/mL) and incubated for six to seven days inside an IncuCyte® ZOOM Live-Cell Analysis System (Essen BioScience), during which live images were acquired every four to six hours to quantify the number of GFP or dsRed positive cells. Results were then converted to percentage of starting seed count and graphed using GraphPad Prism®.

Measurement of antitumor activity in animal models

BALB/cJ mice were implanted subcutaneously in the right flank with 2.5 × 10^5 4T1 or 4T1-MSLN cells co-expressing GFP. When palpable tumors had formed, mice were randomly assigned to different groups based on mean tumor volume (from 50 to 100 mm^3) and were treated once a week with control murine immunoglobulin G1 (mulgG1; 5 mg/kg), anti-MSLN (5 mg/kg) mulgG1, anti-muCD40 1C10 mulgG1 (1.25, 2.5, or 5 mg/kg), or the surrogate bispecific molecule, LB-1(1.25, 2.5, or 5 mg/kg).

To establish a prophylactic PC3 tumor model, immune-deficient NSG mice were inoculated subcutaneously with a mixture of cells including 1 × 10^7 PC3 or PC3-MSLN tumor cells, 1 × 10^6 T cells, and 5 × 10^5 moDCs in 100 µL DPBS. At the time of
inoculation, mice were dosed intraperitoneally (IP) with a mAb against human cytomegalovirus as an isotype control, parental agonistic anti-CD40 or ABBV-428 at 1 mg/kg.

For both prophylactic and syngeneic models, tumor size was measured approximately every 3 to 4 days with electronic calipers, and tumor volumes calculated using the formula: \( V = \frac{1}{2} \times \text{length} \times \text{width} \times \text{height} \). Mice were euthanized when tumor volumes reached a maximum of 2500 mm\(^3\) or if animal health was compromised, per IACUC approved protocol.

**In vivo MSLN expression, liver toxicity, and cytokine production**

To monitor MSLN expression in 4T1-MSLN model, tumor tissues were harvested at randomization and were minced in PBS with a scalpel to obtain ~1-3 mm\(^3\) pieces. Minced tissues were transferred into 50 mL conical tubes and were digested with 10 mL of 0.05% trypsin-EDTA (ThermoFisher) in each tube on a nutating platform (18rpm) at 37 C for 30 minutes. Enzymatic digestion was stopped by adding FBS to 20%. Single-cell suspensions were prepared after carefully triturating and flowing-through a 70\(\mu\)m cell strainer. MSLN expression was measured by flow cytometry analysis on GFP\(^+\) cells after stained by fluorophore conjugated anti-MSLN (clone K1). Soluble MSLN in the blood was measured by ELISA using Max\textsuperscript{TM} Deluxe Set (BioLegend).

Blood samples were collected from mice 24 hours after IP dosing. Direct measurement of alanine aminotransferase (ALT) in blood was performed by comprehensive blood profiling rotor (ABAXIS) per the manufacturer's instructions. Mouse sera were prepared and mouse cytokines/chemokines in 25 \(\mu\)L serum samples were assayed using the MILLIPLEX MAP Mouse Cytokine/Chemokine Multiplex Assay kit (Millipore), measured on a BioRad Bio-Plex\textsuperscript{®} 200 system, and analyzed by Bio-Plex Data Pro Software (BioRad).
Mouse livers harvested at the end of the study were fixed in 10% formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) to assess hepatocyte morphology and tumor metastasis. Immune cell infiltration was evaluated by immunohistochemistry (IHC) staining with anti-CD45 (clone 30-F11) (BD) and rabbit polyclonal anti-IBA1 (Wako) after the antigen retrieval process. The staining was detected using Rat on Mouse or Rabbit HRP-polymer (Biocare Medical) and Envision+ (DAKO) kits accordingly. Images were captured by Aperio AT2 Scanner (Leica) and analyzed by HALO imaging analysis system.

Measurement of immune cell activation in animal models

In a subset of mice, blood, spleen, tumor, and tumor-draining lymph node samples were collected 24 hours after treatment for immune phenotyping. B-cell counts in blood were measured by flow cytometry using Trucount™ tubes (BD Biosciences) per manufacturer's instructions. Draining lymph nodes and spleens were pressed through 100 μm nylon cell strainer (VWR) using the plunger end of a syringe in RPMI1640 with 10% FBS. Tumors were processed using a gentleMACS™ mouse tumor dissociation kit and gentleMACS™ Octo Dissociator with Heaters under 37°C m_TDK-2 program (Miltenyi Biotec). Red blood cells from blood and spleen cell suspensions were lysed with Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich). All murine immune cells collected were blocked with 10 μg/mL mouse BD Fc Block (BD Biosciences) for 15 minutes and stained with fluorophore conjugated antibodies for flow-cytometry analysis as described above.

To measure T-cell responses to recall antigens, mouse spleens were harvested at the end of the study. CD3+ T cells were purified by negative selection using EasySep™ mouse T cell isolation kit (STEMCELL Technologies), and 1 × 10^5 T cells were cultured alone or cocultured for 24 hours with 5 × 10^5 irradiated parental 4T1 or 4T1-MSLN cells in RPMI-1640 with 10% FBS and 50 ng/mL rIL-2 (R&D Systems) in triplicate wells in 96-well U-bottom plates (Sigma-Aldrich). Production of IFNγ was measured by ELISPOT as described above.
**Statistical analysis**

Data were analyzed using GraphPad Prism 7 software. Statistical differences were determined to be significant at $P \leq 0.05$ using one-way (treatment) ANOVA and Duncan’s multiple range tests as appropriate. Data were presented as mean±SEM. In all figures: *$P \leq 0.05$.

**RESULTS**

**Generation of bispecific molecules LB-1 and ABBV-428**

LB-1 is a recombinant bispecific molecule targeting mouse CD40 and MSLN, and ABBV-428 is an equivalent version targeting human CD40 and MSLN. Both LB-1 and ABBV-428 contain scFv domains constructed from the variable regions of anti-CD40 and anti-MSLN as shown in Fig. 1A. Each molecule is composed of a homodimer of two identical chains covalently linked by disulfide bonds. Each chain incorporates CD40 and MSLN targeting scFv domains at the N- and C- termini, respectively. The CD40 and MSLN domains are on the opposite ends of each chain, separated by a hinge region, the Fc part of an antibody, and a short amino acid linker. The calculated molecular weight of LB-1 or ABBV-428 is approximately 160 kDa, slightly larger than a typical IgG antibody having a molecular weight of approximately 150 kDa [33].

Binding of LB-1 or ABBV-428 to CD40 and MSLN was measured by flow cytometry analysis, and results showed that binding of the scFv domains within the bispecific molecule to their target antigens was similar to that of the corresponding parental anti-CD40 and anti-MSLN, except for LB-1 which showed lower binding potency (by EC$_{50}$, half-maximal effective concentrations), but similar maximal binding (by MFI, mean fluorescence intensity) to 1C10, the parental anti-mouse CD40 (Supplementary Table S1, Supplementary Fig. S1). LB-1 contains a mouse IgG1 constant region, and ABBV-428 contains a human IgG1 constant region consisting of a V273E mutation that did not induce antibody-dependent cell-mediated cytotoxicity (ADCC) on CD40$^+$ cells (Supplementary Fig. S1C). Thus ABBV-428 is functionally similar to LB-1 containing the mouse IgG1 backbone.
**LB-1 induces antitumor responses with limited toxicity**

To determine the MSLN-dependent conditional activity of LB-1, mouse B cells were evaluated for activation by LB-1 in the presence or absence of cell surface-expressed MSLN. As shown in Fig. 1B, when B cells were cultured with mouse 4T1 tumor cells without MSLN expression, LB-1 was less effective than parental anti-CD40 (1C10; here on referred to as 1C10) in stimulating B-cell expression of activation markers CD23 and CD86. However, when B cells were cocultured with 4T1 stably overexpressing MSLN (4T1-MSLN), LB-1 was more potent than parental anti-CD40 in activating B cells. Although LB-1 maintained baseline activity in the absence of MSLN, enhanced CD40 activation upon MSLN binding suggests that LB-1 exhibited MSLN-dependent activity.

To test whether the MSLN-dependent activity of LB-1 could provide a better therapeutic index, LB-1 was evaluated in an animal model carrying 4T1-MSLN tumors. Like many MSLN positive human cancers, established 4T1-MSLN syngeneic tumor maintained heterogenous MSLN expression (Supplementary Fig. S2A), and soluble MSLN in circulation could be detected (Supplementary Fig. S2B), probably due to MSLN shedding from tumor cells.

In 4T1-MSLN tumor model, LB-1 was as potent as 1C10 in inhibiting tumor growth when dosed at 2.5 or 5.0 mg/kg, whereas anti-MSLN or a control antibody with the same mouse IgG1 isotype had no anti-tumor activity (Fig. 2A). Antitumor activity was not further enhanced when treated with an 1C10 and anti-MSLN combination (Supplementary Fig. S2C). Although both 1C10 and LB-1 showed antitumor activity at the dose of 5 mg/kg, 1C10 resulted in elevated serum ALT, whereas LB-1 maintained the same ALT concentrations as the control (Fig. 2B). Livers were harvested at the conclusion of the study to evaluate inflammation by IHC staining for CD45 (pan-immune cell marker) and IBA1 (ionized calcium-binding adaptor molecule-1, a pan-macrophage marker, as well as an activation marker). Increased parenchymal and perivascular CD45+ and IBA1+ cells in the livers were observed in
the 1C10-treated mice compared to isotype control-treated mice. In contrast, livers from LB-1–treated mice did not show increased inflammation and had similar basal staining in the parenchyma as animals treated with control antibody (Fig. 2C-D, Supplementary Fig. S3A-B). Serum cytokines were also monitored 24 hours after dosing (Fig. 2E). Cytokines, including IL6, TNFα, KC (CXCL1), IP-10 (CXCL6), and MIG (CXCL9), were significantly elevated in mice dosed with 1C10 at 5 mg/kg. However, at the same dose, LB-1 did not enhance cytokine release in the circulation compared to the control.

In the 4T1 tumor model without MSLN expression, LB-1 did not show antitumor activity or systemic toxicity (Supplementary Fig. S4), suggesting that LB-1 only had antitumor activity in mice carrying MSLN+ tumors. The systemic presence of soluble MSLN in 4T1-MSLN model had minimal impact on the conditional activity of LB-1. Taken together, these data suggest that LB-1 could achieve antitumor potency with reduced systemic toxicity in a tumor model expressing cell surface MSLN.

**LB-1 demonstrated enhanced immune activity within tumor**

To understand the mechanisms mediating the antitumor potency with limited toxicity by LB-1 in vivo, immune cell activation was monitored on cells isolated from blood, spleen, draining lymph nodes, as well as tumors, 24 hours after dosing. In Fig. 3A, more than 90% reduction of peripheral B cells (B220+) in the blood was observed in the mice treated with 1C10. B-cell reduction by LB-1 was about 50% compared to the 1C10 group, probably due to the lower baseline activity of LB-1 as indicated by in vitro assays (Fig. 1B). The in vivo activity of LB-1 was further compared to 1C10 on B cells, as well as on myeloid-derived suppressive cells (MDSCs; CD11b+Ly6G+Ly6Clow). Treatment with 1C10 enhanced activation marker CD86 expression on B cells from the spleen and tumor-draining lymph nodes. LB-1 treatment induced less B-cell activation in the spleen compared to 1C10 (Fig. 3B). However, within tumor-draining lymph nodes, LB-1 was as effective as 1C10 in upregulating CD86 on B cells (Fig. 3C). The differential activity of LB-1 was also observed on MDSCs from circulation versus tumors. In the circulation, the proportion of MDSCs within CD45+ cells was reduced
by 1C10, but not by LB-1 (Fig. 3D). Within tumor, the proportion of MDSCs was reduced by both LB-1 and 1C10 treatment (Fig. 3E). These data suggest that LB-1 was less active in peripheral tissues with limited MSLN expression and was as potent as 1C10 within tumor microenvironment after engaging with MSLN.

To evaluate whether LB-1 could influence the generation of tumor-specific T cells in vivo after 4 weeks with weekly treatment, CD3+ T cells isolated from spleens were cocultured with irradiated 4T1-MSLN or 4T1 parental cell lines, and IFNγ secretion was measured by ELISpot. As shown in Fig. 3F, T cells from both LB-1- and 1C10-treated mice produced more IFNγ than did T cells from control-treated mice in all groups. Upon re-challenge with 4T1-MSLN cells, T cells from LB-1 treated mice produced more IFNγ than those from 1C10-treated mice. When re-challenged with parental 4T1 cells without MSLN expression, T cells from both LB-1 and 1C10 treated mice enhanced IFNγ production similarly. These data suggest that LB-1 could initiate T-cell responses against MSLN, as well as other antigens on the tumor cells. Thus, the antitumor immune response induced by LB-1 within the tumor microenvironment was not restricted to MSLN.

**ABBV-428 exhibits MSLN-dependent activity in vitro**

Having demonstrated that LB-1 with conditional CD40 activity could achieve antitumor potency with limited toxicity in an animal model, we generated ABBV-428, a therapeutic candidate with specificity to human CD40 and MSLN. The conditional activity of ABBV-428 on CD40 activation was tested in human B cells (Fig. 4A) and moDC (Fig. 4B) cultured in the presence of HEK293 cells with or without MSLN-expression. Consistent with known CD40 biology [1-3], agonistic anti-CD40 induced B-cell and moDC activation, whereas ABBV-428 induced minimal B-cell activation and undetectable IL12p70 production from moDCs in cocultures with HEK293 cells without MSLN expression. In contrast, when immune cells were cocultured with HEK293 cells expressing MSLN, ABBV-428 induced B-cell proliferation and IL12 p70 production from moDCs (Fig. 4A-B, Table 1). The MSLN-dependent activity of ABBV-
428 was further confirmed by measuring the expression of activation markers on B cells and moDCs after treatments (Table 1). In the absence of MSLN, ABBV-428 was significantly less potent than the anti-CD40 in the induction of activation markers, as indicated by a higher EC50. In the presence of MSLN-expressing cells, ABBV-428 elicited significantly increased CD86 and CD83 expression on B cells and CD83 expression on moDCs. The MSLN-dependent activity of ABBV-428 required interaction with MSLN expressed on cell surface, as soluble MSLN was not able to drive activation in the culture without cell surface MSLN (Table 1). While in cultures containing MSLN-expressing cells, soluble MSLN at high concentration (300 nM) reduced activity of ABBV-428, possibly through preventing ABBV-428 from binding to cell surface MSLN. These results demonstrated that binding to cell surface MSLN allowed ABBV-428 to enhance CD40 activation in APCs such as B cells and moDCs.

**ABBV-428 induces T-cell activation and prevents tumor growth**

The physiological consequence of antigen presentation is T-cell activation. Thus, ABBV-428 was tested for the induction of MSLN-dependent T-cell activation. In an *in vitro* coculture system mixing T cells with autologous moDCs and PC3 cells or PC3 cells transfected to express high MSLN (PC3-MSLN<sup>hi</sup>), ABBV-428 increased IFNγ production only in cultures containing PC3-MSLN<sup>hi</sup> cells, whereas anti-CD40 stimulated IFNγ production in both cultures containing either PC3 or PC3-MSLN<sup>hi</sup> cells (Fig. 5A).

The impact of ABBV-428 on tumor growth *in vivo* was evaluated. PC3 cells or PC3-MSLN<sup>hi</sup> cells, together with autologous moDCs and T cells, were inoculated into NSG mice. Mice were treated immediately after inoculation. Tumor growth was monitored over three weeks. Compared to isotype control, ABBV-428 reduced the growth of MSLN<sup>+</sup> PC3 tumors, but not MSLN<sup>−</sup> tumors (Fig. 5B). In contrast, anti-CD40 reduced the growth of both MSLN<sup>+</sup> and MSLN<sup>−</sup> tumors. These data suggest that ABBV-428 could only activate moDCs in a MSLN-dependent manner to stimulate the cytotoxicity of autologous T-cells towards allogenic tumor cells.
The amount of MSLN expression are critical for the efficacy of ABBV-428

Because MSLN expression was required for ABBV-428 activity, we next tested whether different amounts of MSLN expression may differentially impact ABBV-428-induced immune cell activation and subsequent tumor growth inhibition. We, therefore, made several PC3 cell lines stably expressing different amounts of MSLN as determined by quantifying the number of cell-surface molecules and assessed in parallel by immunohistochemistry (IHC) (Supplementary Fig. S5). The cell lines with differing expression of MSLN were named as PC3-MSLN^hi, PC3-MSLN^med, PC3-MSLN^low according to IHC score 4+, 3+, and 2+, respectively. Each of these PC3 MSLN-expressing cell lines were then transfected to express GFP and cultured with moDCs and autologous T cells under different treatments. Cell growth was monitored and analyzed on the fluorescence images collected by IncuCyte®. Similar to anti-CD40, ABBV-428 inhibited growth of PC3-MSLN^hi and PC3-MSLN^med tumor cells (Fig. 6A). Unlike anti-CD40, which inhibited growth of tumor cells independent of MSLN expression, ABBV-428 was not able to inhibit growth of PC3-MSLN^low cells (Fig. 6A). These results suggest that ABBV-428 was active only when MSLN expression was above a certain threshold.

Tumors are heterogeneous, and thus, tumor cells with high or low MSLN expression may be present within the same tumor. We next tested the activity of ABBV-428 in cocultures of moDCs and T cells with a mixture of parental PC3 cells co-expressing dsRed and PC3-MSLN^hi cells co-expressing GFP. Consistent with in vivo data, ABBV-428 inhibited growth of PC3-MSLN^hi cells similarly to anti-CD40 (Fig. 6B), but unlike anti-CD40, ABBV-428 did not restrict the growth of parental PC3 cells (Fig. 6C). When a mixture of PC3 cells and PC3-MSLN^hi cells were cultured with moDCs and T cells, ABBV-428 was able to inhibit the growth of both cell types, independent of MSLN expression (Fig. 6D). The data suggest that ABBV-428 required a certain threshold of MSLN expression for initial immune activation, but MSLN was not necessary as a target for tumor cell killing.
DISCUSSION

LB-1 and ABBV-428 were designed as tumor-targeted, agonistic anti-CD40 bispecific molecules to avoid systemic toxicity when treating solid tumors expressing MSLN. It is postulated that MSLN-dependent CD40 activity could reduce toxicity resulting from CD40 systemic activation [23,24]. MSLN+ tumors, such as mesothelioma, ovarian, and pancreatic cancers, usually have a microenvironment associated with a more suppressive phenotype containing few tumor-infiltrating lymphocytes (TILs) [34,35]. Direct activation of CD40 might be able to reprogram suppressive phenotypes, enhance antigen presentation, and elicit subsequent T-cell responses [36-40]. Therefore, MSLN-targeted CD40 therapy may have potential to restore immune responses against those tumors which might not respond well to therapies directly targeting T cells.

LB-1 and ABBV-428 were selected from a panel of molecules with different bispecific formats through in vitro assays for conditional activity. Proof-of-concept studies using LB-1 provided in vivo evidence of the conditional activity which improved the therapeutic index. Similar to LB-1, ABBV-428 also demonstrated in vitro conditional activities and was expected to achieve tumor antigen-driven CD40 activation to improve the safety profile while maintaining potent anti-tumor activity as a therapeutic candidate.

The lower baseline CD40 activity of LB-1 and ABBV-428 in the absence of MSLN cannot be explained by lower binding potency on CD40, although here LB-1 showed lower binding on murine CD40 and ABBV-428 showed similar binding to human CD40 as parental anti-CD40. The molecular structure of LB-1 or ABBV-428 is, therefore, hypothesized to mediate conditional activity. LB-1 and ABBV-428 contains scFv domains constructed from the variable regions of an agonistic CD40 mAb and anti-MSLN. In functional assays, the CD40 scFv domain of the bispecific molecule was less effective than an intact CD40 antibody in initiating CD40 signaling in the absence of MSLN. The structural basis for conditional activation may be related to the structural aspect of CD40 signaling. CD40 belongs to the TNF receptor super family...
Receptors within this family need to be oligomerized to initiate intracellular signaling [41]. An intact antibody which binds to CD40 in a bivalent manner potentiates CD40 multimerization, required for CD40 activation. It is possible that a CD40 targeting scFv domain within LB-1 or ABBV-428 might bind to CD40 in a different geometry from the bivalent binding of a conventional antibody, such that the bispecific molecule needs to bind simultaneously to cell surface MSLN to establish cross-linking, which would in turn multimerize the CD40 molecules and activate downstream signaling. This idea was supported by our observation that the molecule could only achieve enhanced CD40 activation when it bound to cell surface MSLN not soluble MSLN. In fact, soluble MSLN at high concentrations (300 nM) reduced conditional activity by preventing the bispecific molecule from binding to the cell surface MSLN. Because MSLN shedding is common in many MSLN+ tumors, soluble MSLN can be detected in patient serum [26]. Although soluble MSLN from patients is usually less than 3 nM [42], and the antigen sink effect of soluble MSLN was not observed in our preclinical model, optimal dose of ABBV-428 should be explored in future studies in order to overcome the effect of soluble MSLN.

With the MSLN-dependent activity, LB-1 demonstrated antitumor potency with limited systemic toxicity in mice carrying subcutaneous 4T1-MSLN tumors. Subcutaneous 4T1 tumor cells can metastasize to lung and liver [43], and the lower concentrations of liver enzymes and cytokines induced by LB-1 compared to CD40 mAb in our study suggests that LB-1 maintained tumor-specific CD40 activation with minimal damage to adjacent healthy tissue in the organ with metastasis. To further confirm the tumor-specific activity of LB-1, additional in vivo studies will be performed using an orthotopic tumor model or a genetically engineered mouse model which carries spontaneous tumor and organ metastasis.

Although ABBV-428 did not cross-react with mouse CD40 and could not be evaluated in a syngeneic tumor model for conditional activity, ABBV-428 was tested in multiple in vitro assays for functional activity upon MSLN binding. The activity of ABBV-428 was associated with MSLN expression. In moDC/T-cell/tumor cell cocultures, ABBV-
428 showed better tumor cell growth inhibition if tumor cells expressed higher MSLN. Higher MSLN expression ensured the functional enhancement of ABBV-428, even when only a subset of cells expressed MSLN. When MSLN expression was low, even if all cells were MSLN+, ABBV-428 was not able to drive CD40 activation. This allows ABBV-428 to maintain lower CD40 activation within normal tissues where MSLN expression is low, and to initiate more potent CD40 activation in tumors with higher MSLN expression. Understanding the relationship between ABBV-428 activity and MSLN expression is crucial for identifying which patients would be most likely to respond to such therapy.

Although high MSLN expression was required for ABBV-428 to initiate immune cell activation, ABBV-428-mediated tumor cell growth inhibition was not limited to MSLN+ cells. We observed that activated immune cells stimulated by ABBV-428 could inhibit the growth of either MSLN+ or MSLN-tumor cells, as long as the cells with high MSLN expression were present in the mixed cell culture. This phenomenon was confirmed with LB-1, the surrogate of ABBV-428. LB-1 inhibited growth of 4T1-MSLN syngeneic tumors that contained both MSLN+ or MSLN− 4T1 cells. T cells from mice carrying 4T1-MSLN tumors treated with LB-1 also produced IFNγ in response to both MSLN+ or MSLN− 4T1 cells in an ex vivo antigen recall assay. This indicated that MSLN expression may only be required for CD40 activation, and once activated, immune cells can limit tumor cell growth independent of MSLN expression. This feature is different from other tumor-targeting strategies, such as dendritic cell-targeted delivery of MSLN, where MSLN functions as a vaccine [44], or T-cell redirected anti-CD3 bispecific molecules, which only kill target-positive tumor cells upon direct target binding [18,19]. Unlike a vaccine strategy or CD3-mediated T-cell activation, CD40 activation by ABBV-428 or LB-1 within the tumor microenvironment after engaging with MSLN has the potential to initiate immune responses against a broader array of tumor-associated antigens.

The immune memory against MSLN− 4T1 cells observed in our animal studies was less likely due to the baseline CD40 activity of the bispecific molecule, or due to the
lower expression of MSLN on normal tissues. In mice carrying 4T1 tumors without MSLN stable expression, LB-1 treatment did not reduce tumor growth or systemic toxicities. These data again confirmed that the lower expression of MSLN was not sufficient to trigger robust immune activation by LB-1 treatment.

In summary, tumor-targeting anti-CD40 bispecific molecules, such as ABBV-428 and its surrogate LB-1, can achieve tumor-targeted CD40 activation with the potential to improve therapeutic widow. The preclinical data from the current study warrants further clinical development of ABBV-428 and supports testing additional bispecific molecules targeting different antigens with tumor specificity to achieve therapeutic activity across different solid tumor indications.

ACKNOWLEDGMENTS
This study was supported by AbbVie Inc. The authors thank AbbVie Biotherapeutics Inc Animal Facility for animal care support, FACS Core Facility for cell sorting, Protein Science Group for protein expression and purification, and Susan Rhodes from Molecular Cloning Group for generating all constructs used in the studies.

REFERENCES
1. Stamenkovic I, Clark EA, Seed B. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J 1989; 8:1403-10.


Table 1. Summary of Cellular Assays for ABBV-428 Showing MSLN-Dependent Enhancement of Immune Cell Activations

<table>
<thead>
<tr>
<th>Agonistic Assay</th>
<th>Assay Read-Out</th>
<th>−MSLN (EC_{50}, nM)</th>
<th>+MSLN (EC_{50}, nM)</th>
<th>+sol MSLN (EC_{50}, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-cell (n=5)</td>
<td>CD86</td>
<td>0.05 ± 0.18</td>
<td>0.08 ± 0.07</td>
<td>1.2 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>CD83</td>
<td>0.12 ± 0.08</td>
<td>0.17 ± 0.16</td>
<td>8.0 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>Proliferation</td>
<td>1.5 ± 1.2</td>
<td>1.25 ± 1.04</td>
<td>0.06 ± 0.09</td>
</tr>
<tr>
<td>moDC (n=5)</td>
<td>CD83</td>
<td>0.27 ± 0.32</td>
<td>0.09 ± 0.06</td>
<td>0.13 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>IL12 p70</td>
<td>0.77 ± 0.30</td>
<td>0.54 ± 0.14</td>
<td>0.06 ± 0.09</td>
</tr>
</tbody>
</table>

B cells and moDCs from human PBMCs were cultured alone (−MSLN), with MSLN-expressing cells (+MSLN), or with soluble MSLN (+sol MSLN; 300nM). Read-outs of in vitro activity were monitored and compared to an agonistic anti-CD40. EC_{50}: half maximal concentration, ∞: infinite.

FIGURE LEGENDS

**Figure 1. LB-1 enhances B-cell activation in the presence of cell surface-expressed MSLN.**

A, Schematic diagram of our tumor-targeted bispecific molecule. LB-1 (or ABBV-428) is a bispecific molecule with a scFv domain targeting CD40 at N-terminus and a scFv domain targeting MSLN at C-terminus flanking mouse IgG1 Fc (or human IgG1 Fc carrying V273E for ABBV-428). B, B cells were cocultured with 4T1 cells without MSLN expression (4T1) or with MSLN expression (4T1-MSLN), and treated with 1C10, LB-1, or an irrelevant murine antibody as negative control. Expression of CD23 and CD86 on B cells was measured by flow cytometry 48 hours later, indicated by median florescence intensity (MFI). Data are representative of three independent experiments.

**Figure 2. Tumor-targeted bispecific molecule LB-1 demonstrates antitumor efficacy with limited toxicity in a mouse syngeneic tumor model.**

BALB/cJ mice with established MSLN-expressing 4T1 tumors were dosed i.p. with LB-1, anti-muCD40 1C10, anti-MSLN, or an irrelevant murine antibody as control once every week at the indicated doses. A, Antitumor efficacy was assessed by measuring tumor volumes from each group. For each data point, mean and SEM are plotted. Statistical difference was calculated by one-way ANOVA, and presented as *P≤0.05 (n=8). Data are representative of two independent experiments. B, Systemic toxicities were monitored by measuring circulating liver enzyme ALT 24 hours after dosing. Data are representative of three independent experiments and
were plotted with mean and SEM. Statistical difference was calculated by ANOVA with Duncan’s multiple range test and presented as *P≤0.05 (n=5).

C, D At the end of the study, liver inflammation was monitored by IHC for parenchymal and perivascular CD45+ leukocytes and quantified by HALO imaging analysis. Data were plotted with mean and SEM. Statistical difference was calculated by ANOVA with Duncan’s multiple range test and presented as *, P ≤ 0.05 (n=4).

E, Cytokine and chemokine release was monitored by measuring IL6, TNFα, KC, IP10, and MIG in the circulation 24 hours after dosing. For each bar, mean and SEM were plotted. Statistical difference was calculated by ANOVA with Duncan’s multiple range test and presented as *, P ≤ 0.05 (n=8).

**Figure 3.** LB-1 induces tumor-specific immune activation. BALB/cJ mice with established MSLN-expressing 4T1 tumors (n=10) were dosed i.p. with LB-1, anti-muCD40 1C10, or control (Ctrl) antibody once weekly at 5 mg/kg. 24 hours after the first dose, 5 animals per group were sacrificed. B cells in the (A) blood were counted, and (B) B220+ B cells from spleen and (C) draining lymph nodes were assessed for expression of CD86 by flow cytometry analysis, indicated by MFI. D, E, Myeloid-derived suppressive cells (MDSCs) (CD11b+Ly6G+Ly6C<ref>) from (D) blood and (E) tumor were quantified and indicated as percentage of total CD45+ cells collected. F, At the end of study (Day 31), T cells were purified from spleen and either cultured alone or re-challenged with 4T1 or 4T1-MSLN cells in the presence of 50 ng/mL of IL2 for 24 hours. T-cell activation was measured by counting colonies of ELISPOT representing IFNγ secretion. The data shown are representative from 3 individual studies. For each bar, mean and SEM were plotted. Statistical difference was calculated by ANOVA with Duncan’s multiple range test and presented as *, P ≤ 0.05 (n=5).

**Figure 4.** ABBV-428 enhances B cell and moDC activation through binding to cell surface-expressed MSLN. A, B cells purified from human PBMCs were cocultured with HEK293 cells (B cell+293, left) or 293 cells expressing MSLN (B cell+293-MSLN, right). B-cell proliferation was measured by 3H-Thymidine incorporation after treatment with ABBV-428, anti-CD40, or an irrelevant human antibody as control. B, DCs derived from purified human monocytes (moDCs) were cocultured with HEK293 cells (moDC+293, left) or 293 cells expressing MSLN (moDC+293-MSLN, right). IL12p70 production from moDCs was measured in supernatant after treatment with ABBV-428, anti-CD40, or control antibodies. Data shown are representative of 10 donors.

**Figure 5.** ABBV-428 induced T cell activation and inhibited growth of PC3 tumor upon MSLN binding. A, T-cell activation was monitored by coculturing T cells with autologous moDCs and PC3 cells without MSLN expression (T+moDC+PC3, left), or PC3 cells with high MSLN expression (T+moDC+PC3-MSLNhi, right). T-cell activation indicated by IFNγ production was measured by ELISpot 48 hours later. For each data point, mean and SEM were plotted. B, Antitumor activity of ABBV-428 was monitored in a prophylactic tumor model established in immune-deficient NSG mice through subcutaneous inoculation of a mixture of cells including T cells, moDCs, and PC3 (left) or PC3-MSLNhi (right) tumor cells.
At the time of inoculation, mice were administered a single intraperitoneal (i.p.) injection of isotype control mAb (control IgG), an agonistic anti-CD40, or ABBV-428 at 1 mg/kg. Tumor growth was monitored every 5 days. For each data point, mean and SEM were plotted. Statistical difference was calculated by one-way ANOVA, and presented as *P≤0.05 (n=8).

Figure 6. Higher MSLN expression ensures pan-tumor growth inhibition mediated by ABBV-428 induced immune activation. Autologous moDCs and T cells were cocultured with PC3 cells with differing expression of MSLN and co-expressing a fluorescence protein. After treatment with ABBV-428 or an irrelevant human antibody as control, tumor cell growth was continuously monitored by the IncuCyte® ZOOM Live-Cell Analysis System, collecting fluorescence images every 4 to 6 hours for 7 days. Cell growth at each time point was indicated as the percentage of starting cell number (#) and was the average from triplicates. A, Growth of PC3 cell lines with different MSLN expression. B, Growth of PC3-MSLN<sup>high</sup> cells in cocultures containing 100% PC3-MSLN<sup>high</sup> cells. C, Growth of PC3 cells in cocultures containing 100% of PC3. D, Growth of PC3-MSLN<sup>high</sup> cells (left) and PC cells (right) in cocultures containing 50% of PC3-MSLN<sup>high</sup> and 50% of PC3. The data are representative from 5 individual experiments with cells from 5 different donors.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Cancer Immunology Research

A BISPECIFIC MOLECULE TARGETING CD40 AND TUMOR ANTIGEN MESOTHELIN ENHANCES TUMOR-SPECIFIC IMMUNITY

Shiming Ye, Diane Cohen, Nicole A. Belmar, et al.


Updated version  Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-18-0805

Supplementary Material  Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2019/08/28/2326-6066.CIR-18-0805.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/early/2019/08/28/2326-6066.CIR-18-0805. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.