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\textit{J Immunol} 2014; 193:5056-5064; Prepublished online 10 October 2014;
doi: 10.4049/jimmunol.1301723
http://www.jimmunol.org/content/193/10/5056

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4-1BB Ligand Activates Bystander Dendritic Cells To Enhance Immunization in Trans

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Expression of the costimulatory receptor 4-1BB is induced by TCR recognition of Ag, whereas 4-1BB ligand (4-1BBL) is highly expressed on activated APC. 4-1BB signaling is particularly important for survival of activated and memory CD8+ T cells. We wished to test whether coexpression of Ag and 4-1BBL by dendritic cells (DC) would be an effective vaccine strategy. Therefore, we constructed lentiviral vectors (LV) coexpressing 4-1BBL and influenza nucleoprotein (NP). Following s.c. immunization of mice, which targets DC, we found superior CD8+ T cell responses against NP and protection from influenza when 4-1BBL was expressed. However, functionally superior CD8+ T cell responses were obtained when two LV were coinjected: one expressing 4-1BBL and the other expressing NP. This surprising result suggested that 4-1BBL is more effective when expressed in trans, acting on adjacent DC. Therefore, we investigated the effect of LV expression of 4-1BBL in mouse DC cultures and observed induced maturation of bystander, untransduced cells. Maturation was blocked by anti-4-1BBL Ab, required cell–cell contact, and did not require the cytoplasmic signaling domain of 4-1BBL. Greater maturation of untransduced cells could be explained by LV expression of 4-1BBL, causing downregulation of 4-1BB. These data suggest that coexpression of 4-1BBL and Ag by vaccine vectors that target DC may not be an optimal strategy. However, 4-1BBL LV immunization activates significant numbers of bystander DC in the draining lymph nodes. Therefore, transactivation by 4-1BBL/4-1BB interaction following DC–DC contact may play a role in the immune response to infection or vaccination. The Journal of Immunology, 2014, 193: 5056–5064.

When naive T cells are stimulated by Ag, engagement of CD28 by its ligands CD80 or CD86 (termed costimulation) lowers the threshold for activation (reviewed in Ref. 1). T cell activation then induces expression of members of the TNFR family, including 4-1BB, which transmits activation and survival signals during subsequent effector and memory T cell stages (reviewed in Ref. 2). The effects of 4-1BB are most pronounced in CD8+ T cells (e.g., systemic administration of agonistic anti–4-1BB Ab to mice expands CD8+ T cells more than CD4+ T cells) (3). Natural regulatory CD4+ T cells are an important exception to this, because they constitutively express 4-1BB (4), as do a new subset of cytotoxic CD4+ T cells (5). This selectivity for CD8+ T cell stimulation may be due to greater expression of 4-1BBL on activated CD8+ T cells compared with CD4+ T cells (6). During infection of 4-1BB ligand (4-1BBL)–deficient mice with influenza or lymphocytic choriomeningitis virus, the CD8+ T cell response is impaired (7). Also, when human T cells are expanded in vitro with HIV (8) or influenza (9) Ags, 4-1BB triggering increases the expansion of CD8+ T cells with polyfunctional cytokine secretion and higher perforin and granzyme A expression.

The duration and severity of infection influence the expression of 4-1BB on T cells and 4-1BBL on APC. For example, 4-1BBL expression is only significantly upregulated in lung monocytes when mice are infected with the lethal influenza A/PRI8 strain and not with the milder A/X31 strain (10). In A/PR8 infection, 4-1BBL is redundant for the primary response but remains essential for secondary T cell expansion upon Ag re-encounter (10). 4-1BBL on APC is capable of “reverse signaling.” Thus, 4-1BBFc or anti–4-1BBL mAb induces proliferation of monocytes and stimulates cytokine secretion (11). Dendritic cell (DC) maturation also can be stimulated through 4-1BBL (12). Activated DC also can express 4-1BB (13), bringing an extra level of complexity. Stimulation of 4-1BB on DC results in secretion of cytokines (13, 14). Administration of the agonistic anti–4-1BB Ab to RAG-1–knockout mice improves the ability of splenic DC from these mice to stimulate the proliferation of T cells (13). This complicates studies examining T cell responses to 4-1BB (e.g., after stimulation by agonistic Ab), because these may occur both directly through T cell 4-1BB and indirectly through DC activation.

The important role of 4-1BB in the CD8+ T cell effector and memory response has led to a number of attempts to harness it for immunotherapy or vaccination. Agonistic 4-1BB Ab promotes effective antitumor CD8+ T cell responses in mice (15, 16), and clinical trials of this approach have been initiated (reviewed in Ref. 17). Engineering tumor cells to express 4-1BB also inhibits tumor growth and induces tumor-specific T cells (18). This approach of localized 4-1BBL expression, rather than systemic agonistic Ab, has the potential to focus the immune stimulation on...
T cells that recognize the tumor Ags. Similarly, 4-1BBL has been expressed, along with Ag, for immunization against infectious disease. For example, superior CD8+ T cell responses against HIV Gag and Pol epitopes are induced by 4-1BBL expression in poxvirus or DNA vaccines in mice (19–21). An intranasal adenoviral vector (AV) coexpressing 4-1BBL and influenza nucleoprotein (NP) generates superior lung CD8+ T cell responses and protection against PR8 challenge than does one expressing NP alone (22). This 4-1BBL AV also enhances human CD8+ T cell recall responses to NP, resulting in greater expansion of NP peptide-specific T cells with upregulated granzyme A, perforin, and cytolytic activity (22).

Hematopoietic cells have been stably transduced with a lentiviral vector (LV) expressing 4-1BBL to generate a stimulatory APC line capable of long-term T cell expansion in vitro for immunotherapy (23). However, the potential of LV expressing TNFR family ligands as vaccines has not been explored previously. LV are currently being tested as vaccine vectors in an initial clinical trial in HIV-infected vaccine recipients (reviewed in Ref. 24). The ability of LV to transduce nondividing APC in situ (25) and their relatively low intrinsic immunogenicity (26) make them ideal vectors to explore the potency of adjuvants (27, 28). In addition, the long-term expression of Ag presentation achieved by LV (29, 30) might benefit T cell memory, because tonic 4-1BB stimulation may sustain memory populations independently of Ag. Therefore, we constructed an LV expressing 4-1BBL and NP and found that this resulted in superior CD8+ T cell responses against NP and greater protection against lethal influenza challenge than did LV expressing NP alone. Our initial hypothesis was that 4-1BB and NP expressed by the same APC would be most effective. However, when we examined the mechanism of action of 4-1BBL expressed by LV, we found that at least part of its effect is to activate adjacent, bystander DC by triggering 4-1BB.

### Materials and Methods

#### Lentiviral production

LV were derived from pHRSIN-CSGW (31) by insertion of a second promoter and transgene downstream of the WPRE element. Vesicular stomatitis virus G glycoprotein–pseudotyped LV were produced by cotransfection of the vector with pCMVR8.91 (Gag/pol) and pMDG (vesicular stomatitis virus G glycoprotein envelope), as previously described (32). An LV coexpressing a short hairpin RNA (shRNA) targeting 4-1BB together with NP was constructed as described previously (33). Supernatants were concentrated by ultracentrifugation at 24,000 rpm through a 20% sucrose cushion. Titers were measured by reverse-transcriptase assay (Roche Diagnostics, West Sussex, U.K.) and quantitative TaqMan PCR (Applied Biosystems, Warrington, U.K.).

#### Preparation and transduction of DC

Murine bone marrow–derived DC (MuDC) were prepared as described previously (34) and transduced on day 4 of culture in RPMI 1640 with 5% FCS and 50 ng/ml GM-CSF at multiplicity of infections ensuring ≥80% transduction. Control DC were matured with 100 ng/ml LPS. Three days after transduction, cells were harvested for staining and analysis by FACS. After FcR blockade, cells were stained with anti-CD11c–allophycocyanin (eBioscience) and anti-mouse PE-conjugated ICAM-1, CD40, (eBioscience), CD80, CD86, or MHC class II (MHCII; BD Biosciences). Abs against 4-1BBL and 4-1BB and blocking Ab TKS-1 were from eBioscience. Cells were gated for analysis on GFP+CD11c+ for transduced groups and CD11c+ for control groups.

#### Immunization and influenza challenge

Female BALB/c mice, 6–8 wk old, were purchased from Charles River Laboratories and kept in pathogen-free conditions. A dose of 50 ng reverse transcriptase was given by s.c. immunization. Intranasal challenges with influenza virus were performed under ketamine and xylazine anesthesia by inoculation of 20 μl viral suspension into each nostril. Following challenge, mice were weighed daily beginning on day 3, and clinical signs of disease were scored. Mice were sacrificed if weight loss exceeded 25%.

The influenza strain used was mouse-adapted A/PR/8/34 (a kind gift from Dr. Mike Whelan, University College London). Virus was titrated by plaque assay in MDCK cell layers inoculated with 100 μl serially diluted samples and incubated for 3 d after overlay with 0.6% agarose (oxoid) in DMEM.
with 2 μg/ml trypsin (Worthington Biochemical). A dose of 2× LD₅₀ corresponded to 2500 PFU/mouse by plaque assay. This gave 100% lethality in control mice.

**Immunooanalysis**

Cells isolated from spleen or lymph node (LN) were either stained immediately to quantify and phenotype NP-specific T cells [NP₁₄₇₋₁₅₅ pentamer-PE (ProImmune, Oxford, U.K.), anti-CD8–allophycocyanin (eBioscience), and anti-granzyme B–allophycocyanin] or stimulated overnight with relevant peptide and brefeldin A (1 μg/ml) in the last 5 h before staining, followed by permeabilization for intracellular cytokine staining (anti-granzyme B–allophycocyanin and anti-IFN-γ–FITC). For restimulation of CD8⁺ T cell responses in culture, the NP₁₄₇₋₁₅₅ TYQRTRALV peptide, a H₂Kᵈ-restricted CD8 epitope, was used. For restimulation of CD4⁺ T cell responses, the peptide NP₅₅₋₇₈ RLIQNSLTIERMVLSAFDERNKY was used. In addition to intracellular cytokine staining analysis of lung CD4 T cells, splenocytes were restimulated for 4 d in the presence or absence of peptide NP₅₅₋₇₈ in RPMI 1640 with 5% FCS. Supernatants were then harvested and frozen for cytokine analysis by cytometric bead array (TH-1/TH-2 FlowCytomix Kit; eBioscience).

**Statistical analyses**

All data were analyzed using the GraphPad Prism v5.0 statistical software package. The statistical test applied to each data set is indicated in the relevant figure legend.
Results

Engineering DC to express 4-1BBL

To constitutively express 4-1BBL on APC, LV were constructed to express 4-1BBL together with either GFP or influenza NP (Fig. 1A). Expression of mouse 4-1BBL was detected by surface staining of transduced 293T cells or MuDC on day 3 after transduction (Fig. 1B). Endogenous 4-1BBL was not detected on mouse DC after 4 d in culture or on mouse DC transduced with a control LV expressing GFP alone (Fig. 1C). Dose-dependent increases in the expression of 4-1BBL were detected after transduction of mouse DC with increasing amounts of 4-1BBL–GFP LV (Fig. 1C).

4-1BBL enhances NP-specific T cell responses and influenza protection

When LV-encoding Ags are injected s.c. they predominantly transduce DC, which are necessary and sufficient for initiating a T cell response to LV-encoded Ag (35–37). To determine whether 4-1BBL enhanced T cell responses against coencoded Ag, BALB/c mice were vaccinated s.c. with 4-1BBL–NP, Null-NP, or saline and sacrificed 14 d later for splenocyte analysis (Fig. 2A). CD4+ and CD8+ T cell responses were assessed by IFN-γ ELISPOT after overnight restimulation with MHCI- or MHC class I-restricted peptides, respectively. This revealed significantly greater CD4+ and CD8+ T cell responses to vaccination with 4-1BBL–NP than with Null-NP (Fig. 2B). Splenocytes also were restimulated in vitro for 4 d with MHCI–restricted peptide, and supernatants were analyzed by cytometric bead array for a panel of 10 cytokines, including IFN-γ, TNF-α, IL-4, IL-10, IL-17, GM-CSF, IL-1α, IL-5, and IL-6. Significantly greater concentrations of TNF-α, IFN-γ, and GM-CSF were found in supernatants in response to MHCI–restricted peptide restimulation in splenocyte cultures from mice vaccinated with 4-1BBL–NP compared with mice vaccinated with Null-NP (Fig. 2C). This resembles the findings of Li et al. (38), who demonstrated increased IFN-γ and GM-CSF secretion by T cells (and reduced Th2 cytokine secretion) in tumor-draining LN following stimulation with anti–4-1BB Ab in vivo.

Fourteen days after s.c. LV immunization, mice were challenged with a 2× LD50 dose of A/PR/8/34 and monitored for weight loss. All mice developed the severe clinical syndrome of weight loss, tachypnea, and hunched posture. However, at day 6, 6 of 10 mice vaccinated with 4-1BBL–NP began to gain weight, which returned

FIGURE 4. 4-1BBL enhances the quality of the CD8+ T cell response when expressed in trans. (A) Mice were immunized at two sites in the same or opposite flanks (denoted by A, B, and C arrows on diagram) with an LV encoding 4-1BBL and influenza NP together or with two separate vectors encoding influenza NP alone and 4-1BBL alone, and then analyzed as described (bottom panel). (B) Mice were immunized at two sites in the same flank (denoted by A and B on the x-axis) and analyzed 14 d later. The percentage of NP(+)CD8+ cells in spleen (top panel), granzymeB+CD8+ cells in spleen (middle panel), and granzyme B+CD8+ cells in the draining LN (bottom panel) are shown. (C) LV expressing NP or NP together with an shRNA targeting 4-1BB were used to transduce MuDC that were activated with LPS (10 μg/ml) for 6 h. 4-1BB mean fluorescence intensity was reduced from 7642 to 1937 by the shRNA. (D) Mice were immunized at two sites in the same or opposite flanks (denoted by A, B, and C on the x-axis) with the LV shown and analyzed 5 d later. The percentage of granzyme B+CD8+ cells in the draining LN from both flanks (mean + SEM). *p = 0.01–0.04, ***p < 0.0009, paired t test. L, left; R, right.
to normal by day 12; 4 mice died. Only 2 of 18 mice survived in the Null-NP group, although both of them regained their lost weight (Fig. 3). Thus, 4-1BBL–NP vaccination confers a significant survival benefit upon lethal influenza challenge compared with an LV expressing NP alone.

4-1BBL enhances Ag-specific CD8 T cell function when expressed in trans

Our working hypothesis was that 4-1BBL expression on the same DC that expressed and presented NP peptides was leading to enhanced T cell responses. To test this, we compared immunization with an LV coexpressing 4-1BBL and NP and immunization with two separate LV: one encoding 4-1BBL and the other encoding NP. Groups of four mice were vaccinated s.c. on one side in two separate sites that drain to the inguinal LN (Fig. 4A). In previous studies, we tracked transduced DC in the LN following s.c. injection (26, 37). Very few DC (<1% of CD11c+ cells in the LN) would have been transduced with either vector at this dose, so the likelihood of dual transduction was very low. LN and spleens were harvested 14 d after immunization, and T cell responses were assessed.

CD4+ ex vivo responses were not significantly different among the four groups of vaccinated mice, and NP147–155 pentamer responses in the spleen were equivalent among all four groups (Fig. 4B, top panel). However, mice receiving 4-1BBL–GFP in trans with Null-NP demonstrated higher granzyme B expression in CD8+ T cells after overnight stimulation with the CD8-restricted peptide than that observed in mice receiving 4-1BBL–NP and Null-GFP in cis (Fig. 4B, middle panel). In the LN, the group of mice receiving 4-1BBL–GFP in trans with Null-NP was the only one to demonstrate significantly greater granzyme B expression upon restimulation (Fig. 4B, bottom panel). These striking data suggested that 4-1BBL expressed on one population of DC was enhancing NP Ag stimulation of T cells by adjacent DC. To test this hypothesis, we injected 4-1BBL–GFP and Null-NP on the same or opposite flanks and examined the NP response after 5 d in the draining LN. Fig. 4D shows that injection on opposite flanks did not lead to stimulation in trans, supporting the idea that direct DC contact was necessary. We then produced an LV expressing an shRNA directly against 4-1BB together with NP, which downregulated 4-1BB by ∼4-fold when tested in DC cultures (Fig. 4C). This vector did not respond to 4-1BBL stimulation in trans when coinjected on the same flank (Fig. 4D), again supporting a mechanism of direct DC interaction.

4-1BBL activates bystander DC in vitro

DC activation by 4-1BBL was shown to occur both by 4-1BBL reverse signaling and by stimulation of 4-1BB. Therefore, we examined the expression of four activation markers (CD40, CD80, CD86, and ICAM-1) on MuDC following in vitro transduction with 4-1BBL–GFP on day 3 of culture, followed by an additional 4 d of culture. Fig. 5 shows that transduction of these DC cultures with a control LV Null-GFP caused a modest level of activation of the GFP+ cells; we showed previously that this was due to TLR3 and TLR7 triggering on DC by the LV, leading to some activation by the LV particle alone (26). Inclusion of the potent NF-κB activator vFLIP caused a marked activation, as we described previously (28), in this case in the GFP+ transduced cells. Strikingly, 4-1BBL–GFP caused a marked and more pronounced DC activation, predominantly in the GFP− untransduced cells.

4-1BBL-induced bystander DC activation is independent of reverse signaling, requires cell–cell contact, and is abrogated by blocking anti–4-1BBL Ab

To investigate the role of potential reverse signaling in DC maturation, we created a truncated mutant lacking the cytoplasmic N-terminal domain, which includes two putative casein kinase II–signaling regions (39). This mutant 4-1BBL was expressed on the cell surface to an equivalent degree as wild-type (WT) (Fig. 6A). The DC-activation assay revealed stronger upregulation of activation markers in the untransduced population with the truncated 4-1BBL (Fig. 6A), similar to that observed for the full-length 4-1BBL (Fig. 5).

Addition of 4-1BBL–GFP–transduced DC to the upper well of Transwell plates did not increase the activation of untransduced DC in the lower well, suggesting that cell–cell contact, rather than

![Figure 5](http://example.com/figure5.png)
a cytokine-mediated mechanism, is necessary for transactivation of DC by 4-1BB (Fig. 6B). Furthermore, addition of anti-4-1BBL–blocking Ab (clone TKS-1) consistently abrogated activation of the untransduced population in these experiments, regardless of whether 4-1BBLTc–GFP or 4-1BBL–GFP was used (Fig. 6C).

Taken together, these data strongly suggest that the DC activation observed in a total population of DC after transduction by 4-1BBL–GFP occurs by forward signaling to untransduced bystander DC. This presumably occurs through 4-1BB receptor expression on mouse DC, but this does not explain the inferior activation of the 4-1BBL–GFP–transduced population. Given that 4-1BB expression was reported to suppress 4-1BBL expression, we postulated that expression of 4-1BBL reciprocally suppresses 4-1BB expression on the same cell, thus rendering the transduced population less responsive to 4-1BBL.

Membrane expression of 4-1BBL downregulates 4-1BB at the cell surface

To examine the relationship between constitutive expression of 4-1BBL and 4-1BB surface expression, MuDC were transduced with increasing quantities of 4-1BBL–GFP, and expression of the ligand and receptor was analyzed 2 d later. Approximately 40% of untransduced mouse DC expressed 4-1BB at day 6 postmatura-
tion. This 4-1BB+/ve group was as equally susceptible to trans-
duction as the 4-1BB−ve group (Fig. 7A), as shown by an equiva-
lent proportion of GFP+ DC. However, following trans-
duction with 4-1BBL–GFP, 4-1BBL/4-1BB double-positive cells were not observed (Fig. 7A). Because 4-1BBL expression increased in proportion to the amount of 4-1BBL–GFP LV added, 4-1BB surface expression was suppressed; however, intracellular 4-1BB showed only a modest decline (Fig. 7B). These data suggest restriction of 4-1BB surface expression by 4-1BBL expression provides a simple mechanism to explain bystander DC activation by 4-1BBL–expressing DC.

4-1BBL transactivation of DC occurs in vivo

To test whether DC transactivation occurs in vivo and, therefore, could explain the action in trans that we saw in vaccination (Fig. 4), we injected groups of four mice in the flank with either 4-1BBL–GFP or Null-GFP and then collected draining inguinal LN. We then examined GFP+ DC in the draining LN at day 4.
in these experiments, <1% of cells were GFP⁺. This revealed both greater numbers of CD11c⁺ MHCII⁺ GFP⁻ cells in the draining LN of mice receiving 4-1BBL versus Null-GFP (data not shown) and higher numbers of DC expressing CD80, CD86, or both (Fig. 8A). Thus, expression of 4-1BBL on a small number of DC appears to recruit many additional DC to the draining LN and to activate them. To further characterize the degree of upregulation of CD80 and CD86, mice were injected, LN from four mice were pooled, and CD11c⁺ve cells were isolated by bead separation. Cells were then stained for CD11c, MHCII, and CD80 or CD86 (Fig. 8B). These data provide a clear rationale for LV-encoded 4-1BBL as an effective adjuvant when expressed in trans with Ag expressed from a second LV vector.

**Discussion**

We reported previously that a number of intracellular activators can improve LV stimulation of antitumor and anti-influenza immune responses (27, 28). We selected 4-1BBL as a novel adjuvant candidate in LV because of its well-documented role in stimulating CD8⁺ T cell effector and memory responses. Following s.c. LV immunization, which targets DC, we indeed found superior CD8⁺ T cell responses against influenza NP when 4-1BBL also was expressed by the LV. Vaccination with 4-1BBL and NP also conferred greater protection against lethal A/PR/8/34 challenge than did NP. This is in agreement with a previous study using intranasal immunization with an AV coexpressing 4-1BBL and NP (22).

Using LV immunization, we also showed that 4-1BBL and NP should be expressed in trans for an optimal CD8⁺ T cell response. We demonstrated that this is because 4-1BBL expressed on DC following LV immunization can activate neighboring, bystander DC expressing Ag. This is in contrast to the expression of 4-1BBL with Gag in a DNA vaccine, in which only the cis configuration is effective (20). However, the immune responses to an HBsAg DNA vaccine are substantially enhanced when a plasmid expressing 4-1BBL in trans is coinjected (40). Also, an AV expressing 4-1BBL is at least partially effective in bone marrow chimeric mice with 4-1BB expression restricted to hematopoietic cells other than DC (21).
T cells, suggesting that it can act via 4-1BB stimulation on APC (22). In fact, despite the widespread experimental use of 4-1BB stimulation to enhance T cell responses to tumors or pathogens, the degree to which these responses are due to 4-1BB expressed on T cells remains unclear. One confounding factor is that 4-1BB-deficient T cells do not behave normally, even in the absence of 4-1BB. They undergo hyperproliferation and reduced cytokine secretion for an unknown reason; therefore, studies in mice lacking 4-1BB on T cells are difficult to interpret (41). Together, these data suggest that both direct stimulation of T cells and activation of APC, such as DC, should be considered when incorporating 4-1BB into immunization strategies.

Clearly, because DC express 4-1BB during an immune response (13), this activation of neighboring DC expressing 4-1BB may play a physiological role. For example, CD8$^+$ lymphoid-resident DC can cross-present Ag transferred from incoming mi-cro DC–DC signaling occurs in this and other scenarios mediated by WT mice (41), showing that 4-1BB expression suppresses 4-1BBL signaling has multiple functions in human dendritic cells during an adaptive immune response. Eur. J. Immunol. 38: 1034–1042.


