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Adjuvantive effects of anti-4-1BB agonist Ab and 4-1BBL DNA for a HIV-1 Gag DNA vaccine: Different effects on cellular and humoral immunity

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ABSTRACT

Plasmid DNA immunizations induce low levels but a broad spectrum of cellular and humoral immune responses. Here, we investigate the potential of co-stimulation through 4-1BB as an adjuvant for a HIV-1 DNA vaccine in mice. We designed plasmid DNAs expressing either the membrane bound or soluble form of 4-1BBL, and compared with the agonistic anti-4-1BB Ab for their ability to adjuvant the Gag DNA vaccine. Both, anti-4-1BB agonistic Ab as well as 4-1BBL DNA enhanced the Gag-specific cellular immune responses. However, in complete contrast to the agonistic Ab that suppressed humoral immunity to Gag, 4-1BBL DNA adjuvanted vaccines enhanced Gag-specific IgG responses. Importantly, the expression of Gag and 4-1BBL from the same plasmid was critical for the adjuvant activity. Collectively, our data suggest that for a HIV-1 vaccine where both antigen-specific cellular and humoral immunity are desirable, 4-1BBL expressed by a DNA vaccine is a superior adjuvant than anti-4-1BB agonistic Ab.

1. Introduction

Despite intense multifarious efforts over the past several years, a successful HIV vaccine still remains elusive. Of the many current vaccination strategies, DNA vaccination is relatively safe and can induce a broad spectrum of cellular and humoral immune responses. Also, problems like pre-existing immunity, as seen with other viral vectors such as Adenovirus type 5 (Ad5), Adeno Associated Virus (AAV) and Modified Vaccinia Ankara (MVA) can be averted with DNA based immunizations. However, DNA alone is a weak immunogen and several strategies are currently under investigation to adjuvant DNA vaccines to increase their potency and clinical utility. One such approach is to modulate and enhance the host immune response by using genetic adjuvants such as cytokines, chemokines and T cell co-stimulatory molecules as a component of the vaccine.

4-1BB (CD137), a type I transmembrane protein, is a member of the TNFR superfamily (TNFSF9) with several extracellular cysteine-rich domains. 4-1BB expression can be induced on CD4 and CD8 T cells upon activation [1,2] and is also found on a subset of splenic and bone-marrow derived DCs, mast cells, natural killer (NK) cells and on human monocytes and eosinophils [3]. Surface expression of 4-1BB on activated T cells is transient, reaching the peak at about 48 h post-activation and declining by 4–5 days [1,4]. Co-stimulation through 4-1BB delivers pro-survival signals during the peak of effector phase of an immune response that is shown to be important for preventing activation induced cell death of the effector population, thus generating a larger memory pool. Consistent with this, several studies have indicated that 4-1BB and 4-1BB ligand (4-1BBL) interactions are important for inducing robust CTL responses and for the establishment of long-lived memory CTLs [5–8]. All of these favorable effects on the final outcome of an immune response make this co-stimulatory pathway an attractive target for immunotherapy and adjuvanticity.

Because of its crucial role in the generation and sustenance of CD4 and CD8 T cell responses, the 4-1BB/4-1BBL co-stimulatory pathway has been exploited both for anti-tumor and anti-viral immunity [9–12]. However, an interesting and important feature of this co-stimulatory pathway is that signaling through 4-1BB can also suppress the immune response. In contrast to its positive regulatory role on T cells and anti-tumor/anti-viral activity, 4-1BB co-stimulation has also been shown to ameliorate or prevent various autoimmune conditions in mice by suppressing immune responses [13–15]. Thus, given its conflicting role on immune regulation, it is important to modulate this pathway appropriately for the purpose of vaccine development where enhancement, and not suppression, of the immune response is desirable.

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In this study, we investigated if the form of adjuvant could influence antigen-specific T and B cell responses elicited by a HIV-1 Gag DNA vaccine in mice. We designed plasmid DNAs expressing either membrane bound or soluble 4-1BBL, and compared with the agonistic anti-4-1BB Ab in their ability to adjuvant the DNA vaccine. Our results demonstrate differential adjuvantive effects on the cellular and humoral immunity by the agonistic Ab and ligand DNA. Our results also highlight that by changing the form (agonistic Ab vs. ligand DNA), the same molecular adjuvant can be exploited either to enhance or suppress the humoral arm of adaptive immunity and thus can have varied applications in anti-tumor or anti-viral vaccines or in autoimmune diseases.

2. Materials and methods

2.1. Immunizations

Female BALB/c mice of 6–8 weeks of age were purchased from Charles River Laboratories (Wilmington, Mass.). The DNA immunogen, pGA1/JS8 [16], expresses clade B consensus Gag. The MVA immunogen, MVA/HIV62 expresses HIV-1 clade B Gag-Pol and Env [17]. Both DNA and MVA vaccines were administered intramuscularly. Agonistic anti-4-1BB Ab (clone 3H3) was given intraperitoneally at a dose of 200 μg per mouse. Unless otherwise stated, for experiments with agonistic Ab, DNA vaccine was used at a dose of 100 μg per mouse. For *in vivo* depletion of CD4 T cells, mice ($n = 3$) were injected i.p. with 200 μg of GK1.5 antibody 1 day before and after DNA prime along with or without the agonistic anti-4-1BBAb. Gag-tetramer-specific CD8 T cells were evaluated in blood for both wild type and CD4 depleted mice at different time points following MVA boost. For experiments with ligand DNA, Gag, m4-1BBL and SPD-4-1BBL DNAs were used at a dose of 20 μg per mouse, and the Gag-m4-1BBL DNA was used at a dose of 40 μg per mouse. MVA vaccine was used at a dose of 10^6 pfu. All immunizations were performed in sterile PBS in a final volume of 100 μl and 50 μl was injected in each of the hind legs. Mice were cared for under guidelines established by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals" using protocols approved by the Emory University Institutional Animal Care and Use Committee.

2.2. Construction of 4-1BBL DNA plasmids

cDNA encoding murine 4-1BBL was PCR amplified using specific primers. The oligonucleotide primers were designed to include NheI site in the sense primer (TATCGCTAGCATGGACCAGCACACACTTGATG) and AvrII site in the antisense primer (TATCCCTAGGTCATTCCCATGGGTTGTCGGG). The restriction enzyme (RE)-digested PCR fragment was ligated to the pGA1 expression vector at NheI and AvrII sites to create the m4-1BBL plasmid (GenBank Accession#: GQ258348). Both the m4-1BBL and the pGA1/JS8 plasmids were digested with NotI and PvuI and the 4-1BBL gene along with the CMV-IA promoter (insert) was ligated to the pGA1/JS8 expression vector to create the 4-1BBL and Gag co-expressing plasmid (Gag-m4-1BBL plasmid, GenBank Accession#: GQ258349). The insertion and orientation of the 4-1BBL cDNA in the expression vectors were confirmed by RE analysis. The SP-D (lung surfactant protein D) fragment was amplified from pSP-D-CD40L [18] using the following PCR primers: sense GGGGGCTAGCGAATTCCACCAGGAAGC; antisense CTCGGTGCGGCCATCAGGGAACAATGCAGC. The extracellular domain (ECD) of 4-1BBL was PCR amplified using CCCTGATGGCCGCACCGAGCCTCGGC (sense) and TATCCCTAGGTCATTCCCATGGGTTGTCGGG (antisense). The PCR products were gel purified and an overlap PCR was done to create the junction between SP-D on the 5′-end and ECD of 4-1BBL on 3′-end. The amino acid sequence at the junction between SP-D and murine 4-1BBL was KAALFPDG/RTEPRPAL, where the N-terminal portion is from SP-D (amino acids 1–256 of GenBank protein sequence no. NP_033186) and the C-terminal portion is the extracellular sequence of murine 4-1BBL (amino acids 104–310 of GenBank protein sequence no. NP_033430.1). The overlap PCR product (SPD-4-1BBL form) was ligated to the pGA1 expression vector between NheI and AvrII sites (GenBank Accession#: GQ258350).

2.3. Protein expression analysis of 4-1BBL

293T cells (human embryonic kidney cell line) were transfected with plasmid DNA using Lipofectamine 2000 following the manufacturer's guidelines (Life Technologies). Intracellullar expression of 4-1BBL and Gag were analyzed by flow cytometry using flurochrome-conjugated mAbs 4-1BBL-FITC (clone 19H3) and KC-57-PE (Beckman Coulter), respectively. To isolate virus-like particles (VLPs), supernatants from plasmid DNA transfected 293T cells were overlayed on a 20% sucrose layer and centrifuged in a Beckman SW41 rotor at 32,000 rpm for 2 h and pellet was resuspended in PBS. VLPs were diluted with sample buffer and were run on 4–15% acrylamide gels (Biorad). Gels were transferred to nitrocellulose membranes and probed sequentially with anti-4-1BBL polyclonal antibody (clone D-20, Santa Cruz Biotechnology), and anti-goat IgG-HRP (Santa Cruz Biotechnology). Western blots were developed using the chemiluminescence reagent (GE Healthcare). For detecting Gag, H12.5C mAb (NIH AIDS reagent resource) was used as the primary antibody and anti-mouse IgG-HRP (Sigma) was used as the secondary antibody. For detecting SPD-4-1BBL in 293T cell culture supernatants, purified anti-mouse 4-1BBL mAb (clone TKS-1) was used as the primary antibody and anti-rat IgG-HRP (Southern Biotech) was used as the secondary antibody. The polyclonal Ab was not used for this purpose, as this Ab was raised against a peptide mapping near the N-terminus of the 4-1BBL protein that had been deleted in the SPD-4-1BBL form. Plasmid DNAs were grown in *E. coli* DH5α and purified using Endotoxin-free Gigaprep kits (Qiagen).

2.4. In vitro activity assay for 4-1BBL

Total splenocytes (7×10^6 cells/well) were stimulated with soluble anti-CD3 (clone 145.2C11, 1 μg/ml) for 72 h at 37 °C in 6-well plates. After 72 h, cells were washed and surface expression of 4-1BB was confirmed by flow cytometry. 96-well plates were coated overnight with anti-CD3 (clone 145.2C11, 0.003 μg/ml) and mouse IgG (1 μg/ml). The 72 h stimulated splenocytes (0.5×10^6 cells/well) were cultured with 50 μl of 293T culture supernatants containing either membrane bound 4-1BBL (m4-1BBL) on VLP or SPD-4-1BBL for an additional 48 h. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) for the last 8–10 h before harvesting and proliferation was measured by scintillation counting.

2.5. Tetramer assays

For tetramer analyses, cells were stained using allophycocyanin (APC)-conjugated Gag-tetramer (NIH tetramer core facility), CD4-FITC, CD19-FITC, CD11a-PE and CD8-PerCP in 100 μl of complete RPMI at 4 °C for 30 min. Cells were washed twice with wash buffer (PBS with 2% FBS) and acquired (approximately 200,000 lymphocytes) on the FACS Calibur (Becton Dickinson) and analyzed using Flowjo software (Tree Star, San Carlos, CA). All antibodies were purchased from BD Pharmingen. CD8+ CD11a+ CD4− CD19− and Gag-tetramer+ cells were scored as tetramer positive cells.