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Incorporation of 4-1BB ligand into an adenovirus vaccine vector increases the number of functional antigen-specific CD8 T cells and enhances the duration of protection against influenza-induced respiratory disease

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ABSTRACT

T cell based influenza vaccines offer the potential for cross protective immunity to multiple clades of influenza virus. Here we explored the effect of increasing CD8 T cell responses during intranasal vaccination by incorporating a T cell costimulator, 4-1BBL. Inclusion of 4-1BBL in an influenza nucleoprotein (NP)-containing adenoviral vector increased the number of NP-specific CD8 T cells and lowered the vaccine dose required for short-term protection from influenza-induced disease in mice. At higher vaccine doses, the inclusion of 4-1BBL increased the duration of protection of mice from influenza-induced mortality. Bone marrow chimera experiments revealed that the major effects of 4-1BBL were directly on αβ T cells with minor additional effects through cells other than αβ T cells. The implications of these findings are that including 4-1BBL or adjuvants that induce 4-1BBL expression may be of benefit in a vaccine setting for enhancing the magnitude and duration of T cell responses to influenza virus.

1. Introduction

Influenza remains an important health concern throughout the world [1]. In part this is related to the annual influenza epidemics that kill thousands [2]. However the threat of pandemic influenza has highlighted the need to have an effective strategy to control this virus [3–5]. Although new and existing therapeutics show some promise for treating existing disease, much emphasis has been placed on preventative strategies and numerous vaccine models are currently being studied. Currently available influenza vaccines generate humoral immunity to epitopes from the influenza surface proteins, neuraminidase and hemagglutinin [6]. Although this strategy can result in sterilizing immunity, these targets change frequently and thus often the vaccine is not a match for the circulating virus.

It is known that CD8 T cells represent an important part of protective immunity to viruses [7]. Studies in humans suggest an important role for cross-protective T cells in recovery from influenza infection [8] and indeed CD8 T cells can transfer protection in mouse models of influenza infection [9] (reviewed in [10]). The nucleoprotein (NP) gene of influenza is relatively conserved compared to other influenza gene products [11,12] and is a major target of cross protective CD8 T cells [13].

Vaccination strategies incorporating NP have demonstrated protection against heterologous influenza strains in mouse models [14,15]. However, other studies have found that NP alone is not optimal for generating protective immunity to influenza [16–19]. Additional strategies to optimize CD8 T cell responses are required as part of a concerted approach to both epidemic and pandemic influenza.

In this study, we examined the effect of increasing the CD8 T cell response on protective immunity to influenza virus by incorporating a costimulatory ligand, 4-1BBL, into a replication defective adenovirus containing the NP gene. Recombinant adenoviral vectors have been utilized in numerous vaccine settings to generate both humoral and T cell immunity. Although concerns have been raised regarding reduced immunogenicity with pre-existing adenovirus immunity, this limitation can be overcome, as discussed in recent reviews [20,21]. 4-1BBL is a TNF family ligand found on activated antigen presenting cells [22,23] that binds to 4-1BB, a member of the TNF receptor family expressed on activated T cells [24,25] as well as on other cell types, such as dendritic cells and natural killer cells (for review see [26]). We chose to incorporate 4-1BBL into our vaccine model based on previous work highlighting

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its role in enhancing the expansion of activated and memory CD8 T cells (reviewed in [26,27]).

We report that inclusion of 4-1BBL with influenza NP in a single dose intranasal adenoviral vaccine results in enhanced protection to severe influenza challenge, correlating with an increased CD8 T cell response. These studies illustrate that targeting the 4-1BB costimulatory pathway is a viable strategy for improving CD8 T cell based influenza vaccines.

2. Materials and methods

2.1. Mice

Wildtype (C57BL/6) mice were obtained from Charles River Laboratories (St-Constant, Quebec, Canada). 4-1BB-deficient (4-1BB$^{-/-}$) mice on the C57BL/6 background (obtained from Dr. B. Kwon [28]) were bred under specific pathogen free conditions at the University of Toronto. All mouse experiments were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on Animal Care.

2.2. Adenoviral vectors and immunization

Replication defective adenovirus 5 (AdV) expressing influenza NP in the E1 region was kindly provided by J. Bramson (McMaster University, Hamilton, ON, Canada). The NP was derived from influenza A/PR8 GenBank: J02147.1. AdV expressing NP in the E3 and 4-1BBL in the E1 region was generated as follows: NP was first cloned into pcDNA3.1. Then the whole NP expression cassette including a CMV promotor and BGHpolyA of pcDNA3.1 was subcloned into the PacI site of the E3 region of AdV genomic plasmid, pBHGloxDel-E1, 3-cre2. 4-1BBL cDNA was then cloned into the polylinker region of the AdV shuttle plasmid pDC315 (driven by the mouse CMV promotor). Next, both the AdV genomic plasmid with NP and the shuttle plasmid with 4-1BBL were co-transfected into 293 cells to generate a replication-defective adenovirus expressing both NP and 4-1BBL. 293 cells were infected for large scale virus production with cesium chloride gradient ultracentrifugation for purification and plaque assay for quantification. For intranasal and intramuscular immunization, age-matched male mice of between 6 and 10 weeks of age were anaesthetized with isoflurane. 30 μL of diluted virus was given either via nares or injected into the right thigh.

2.3. Viruses, infections and titers

Influenza A/PR8 was grown in eggs and tissue culture infectious dose determined by infection of MDCK cells [29]. For intranasal infection, age-matched male mice of between 6 and 10 weeks of age were anaesthetized with isoflurane. For primary intranasal (i.n.) infection with Influenza A/PR8, 30 μL of diluted virus was given. For each viral preparation, the viral dose was titrated in unimmunized mice and 1 $LD_{50}$was used for all challenge experiments. Mice were monitored closely with daily weights and body temperature readings (rectal temperature) and euthanized when moribund.

2.4. Lung, spleen, blood and mediastinal lymph node lymphocyte harvest

Organs were removed from mice and resuspended in HBSS after passage through a 70 μM filter with the aid of a 5 mL syringe plunger. The lungs were perfused (via the right ventricle) with 5–10 mL of PBS to remove cells in the pulmonary vasculature. Spleen and lung lymphocyte suspensions were hemolysed by a 2-min incubation with ACK lysis buffer. In addition, lung lymphocytes from individual mice were then enriched by isolation over an 80/40% percoll gradient. Blood was collected from mouse saphenous veins using a heparin coated tube. After RBC lysis, the cell suspensions were used for tetramer analysis and *ex vivo* peptide restimulation assay.

2.5. Flow cytometry

Influenza $NP_{366–374}$-specific CD8 T cells were enumerated using MHC tetramers (obtained from the NIAID tetramer facility, Emory University, Atlanta, Georgia), as previously described [30]. Samples were analyzed using a FACScalibur (BD Biosciences) and FlowJo (TreeStar Inc., Ashland, OR, USA) software. Where indicated, cells were labeled with antibodies to CD107α, IFNγ, (BD Biosciences) or CD8 (eBioscience).

2.6. Cytotoxicity assay

CTL activity was measured in an *ex vivo* CTL assay as previously described [31] after a 5-day stimulation with 0.1 μM $NP_{366–374}$ peptide. The percentage of specific lysis was calculated using the following equation: 100% × (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release) = % specific lysis.

2.7. T cell isolation and adoptive transfers

CD8 T cells were purified from spleens of immunized mice using a negative selection mouse CD8 T cell enrichment kit (StemCell Technologies), and injected i.v. into mice 24 h before infection with influenza A/PR8.

2.8. Generation of mixed bone marrow chimeras

Bone marrow cells were obtained from femurs and tibia of WT, TCRα$^{-/-}$ and 4-1BB$^{-/-}$ mice. 4-1BB$^{-/-}$ mice were irradiated with two doses of 550 rad, followed by reconstitution with a 4:1 ratio of TCRα$^{-/-}$ to WT or 4-1BB$^{-/-}$ bone marrow cells through intravenous injection with 5×10^6 cells in a volume of 200 μL. This 4:1 ratio ensures that the majority of non-T cells transferred are competent in expressing 4-1BB in the TCRα$^{-/-}$: 4-1BB$^{-/-}$ bone marrow chimeras. Reconstituted mice were given water supplemented with 2 mg/mL neomycin sulfate (Bio-Shop, Burlington, ON, Canada) during the first 4 weeks, and then were rested for an additional 2 months before intranasal immunization.

2.9. Statistical analysis

Where indicated, *p* values were obtained using one-way ANOVA to compare multiple groups, with either Tukey's test or a Bonferroni correction. Statistical significant differences were as indicated or reported as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. Student's *t*-test was used to compare pairs of groups (unpaired, 2 tailed), expect for comparison of weight and temperature post infection where a paired *t*-test was used. A logrank test was used for analysis of the survival curve (95% confidence interval). Prism software was used for all analysis (Graphpad Software, La Jolla, CA, USA).

3. Results

3.1. Dose-dependence of intranasal delivery of adenoviral vector expressing influenza nucleoprotein (NP) for generation of NP-specific CD8 T cells

We chose an adenoviral based vaccine model for testing the effect of 4-1BBL on vaccination, because adenoviral based vaccines