Lysosomal-associated membrane protein-1-targeting of Epstein–Barr virus nuclear antigen 1 (EBNA1) elicits potent immune responses and inhibits tumor growth in preclinical studies

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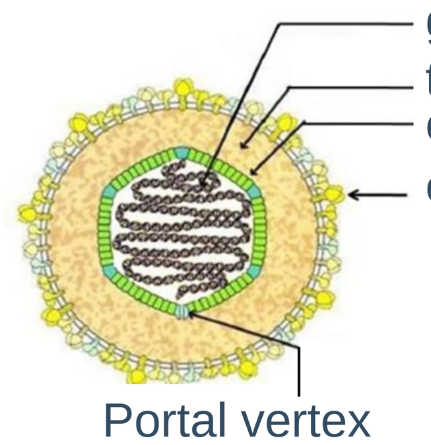
Abstract

Background: Epstein-Barr virus (EBV) is an oncogenic γ-herpes virus that infects >90% of the global population. EBV is the primary cause of infectious mononucleosis and is associated with several epithelial and lymphoid malignancies, including nasopharyngeal carcinoma, gastric carcinoma, non-Hodgkin's and Hodgkin's lymphoma. Of these, nasopharyngeal carcinoma is particularly prevalent in East and Southeast Asia and is highly metastatic. Current treatment strategies for nasopharyngeal carcinoma are limited to radiation and chemotherapy, demonstrating a need for new, targeted therapies. Most nasopharyngeal carcinomas express EBNA1, a DNA-binding protein involved in maintenance of the episomal virus genome that is required for EBV latency and associated transformation. Targeting EBNA1 allows for an immunotherapeutic approach, by exploiting the potential of the immune system to recognize tumor cells through their expression of this viral antigen.
Methods: We designed a DNA vaccine encoding EBNA1 using the UNITE (UNiversal Intracellular Targeted Expression) platform. The UNITE platform is based in part on lysosomal targeting technology which results in enhanced antigen presentation and a balanced T cell response.
Results: We report that the EBNA1-UNITE vaccine induced IFNγ-producing effector memory (EM) $CD4^+$ T and $CD8^+$ T cells with complete rejection of EBNA1-expressing tumors observed in 50% of mice. Mice rejecting tumors were protected from rechallenge with CT26-EBNA1, demonstrating that antigen-specific memory was induced in these animals. Tumor microenvironment (TME) analysis showed vaccine-induced mobilization of effector cells, including tumor infiltration of IL-12-producing type 1 dendritic cells, iNOS-producing M1 macrophages, activated EM $CD4^+$T, and $CD8^+TNF\alpha^+$T cells. Furthermore, increased $PD\text{-}1^+CD8^+$T cells in the TME suggests that a combination strategy with PD-1/PD-L1 blockade may be beneficial in a therapeutic setting.
Conclusions: This pre-clinical data suggests that the EBNA1-UNITE vaccine has the potential to be used as an immunotherapeutic agent against EBV-associated cancers.

Introduction

Epstein-Barr Virus is the most common and persistent virus infection in humans

- approx 95% of the world's population sustaining an asymptomatic, life-long infection
- EBV infection is globally associated with various malignancies such as post-transplantation lymphoproliferative disease (PTLD), Hodgkin/non-Hodgkin lymphoma (HL/NHL), nasopharyngeal carcinoma (NPC), and gastric carcinoma



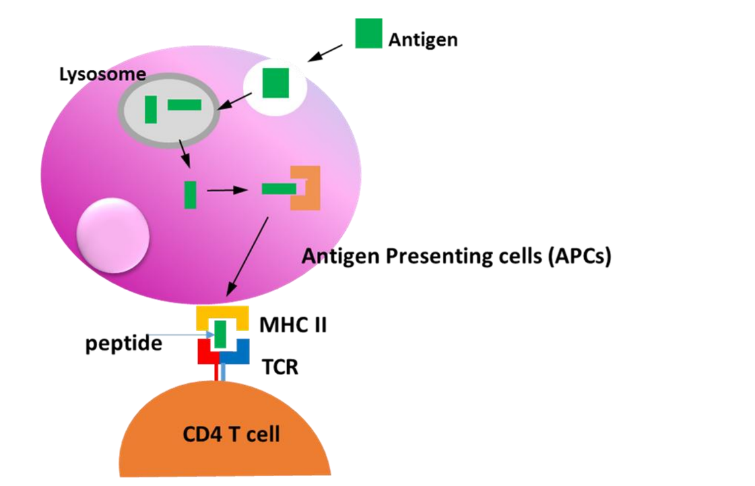
envelope proteins	Receptors	Function
gp350	CR2 (CD21)	attachment to cells
gb	unknown	fusion
gp42	MHCII	fusion with B cells
gH/gL	αvβ6, αvβ8 integrin	fusion with epithelial and B cells
BMRF2	integrins	attachment to epithelial cells

Modified from Chen et al., 2012

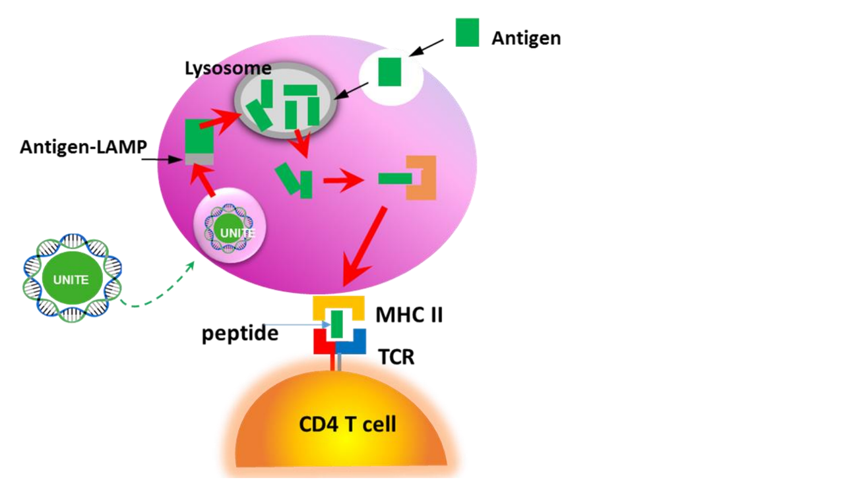
Introduction

- UNITE™-VAX utilizes plasmid DNA expressing TAA and LAMP to deliver TAA to the MHC II compartment, enhancing antibody generation and $CD4^+$ T cell responses.
- Based on our previous studies, we hypothesize that UNITE™-VAX can rapidly prime and activate antigen-specific $CD4^+$ T cells and these activated $CD4^+$ T cells play an essential role for the function and infiltration of $CD8^+$ T cells into tumor sites.

UNITE™ platform: Unique Mechanism of Action



Classical activation of $CD4^+$ T cells is initiated by antigen-presenting cells that process extracellular antigen and present it through MHC class II



TAA-LAMP DNA-mediated activation of $CD4^+$ T cells is achieved through processing of endogenous antigen by antigen-presenting cells

EBNA1-UNITE vaccine construct, in vitro and in vivo validation

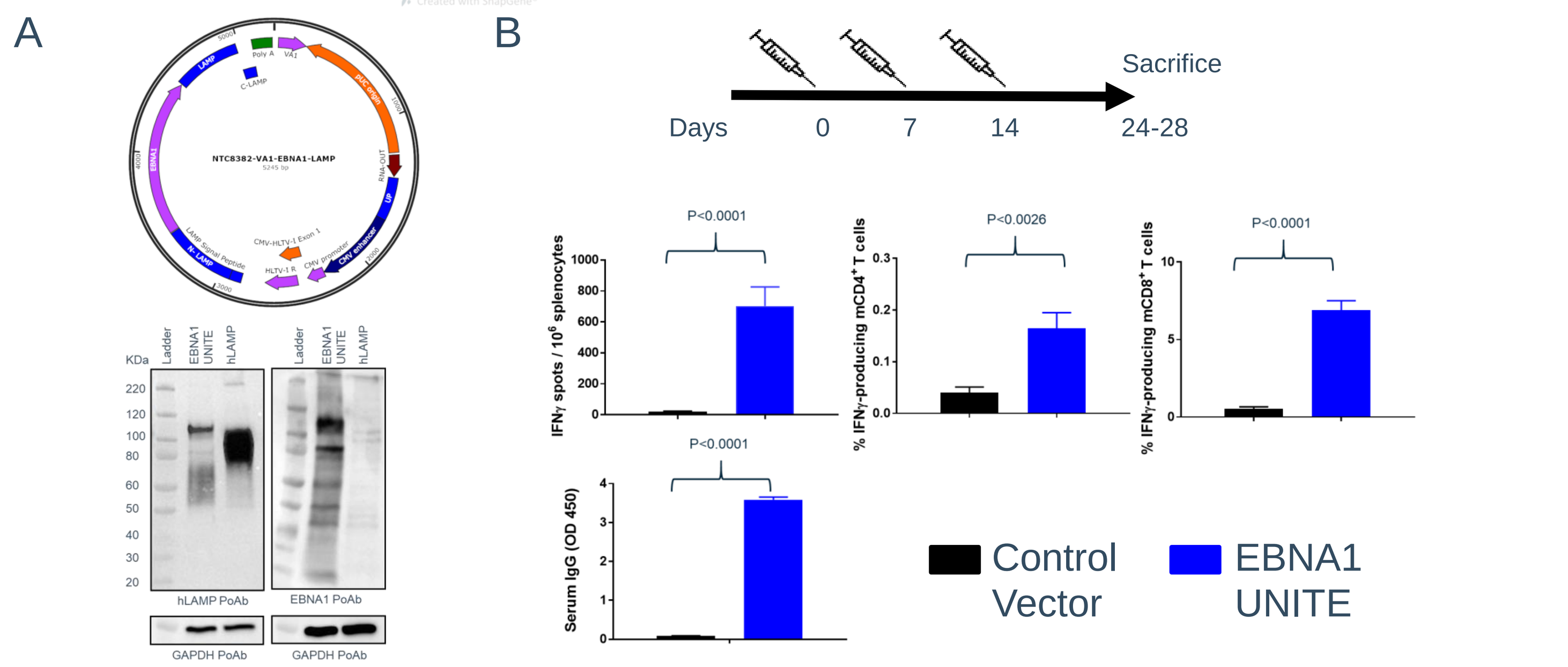


Figure 1: A. Top, Schematic representation of the EBNA1-UNITE vaccine with EBNA1 cloned into the NTC8382-VA1-LAMP plasmid. Bottom, Western blots showing the expression of vaccine antigens. GAPDH was used as loading control. **B.** Top, Vaccination schedule. BALB/c mice were immunized with the vaccine by i.d. delivery in ear and electroporation on days 0, 7, and 14. Mice were euthanized on day 24-28 and blood and spleen were collected. Middle, EBNA1-specific cellular immune response of vaccinated mice was measured by IFNγ ELISPOT and intracellular flow cytometry. Bottom, EBNA1-specific humoral immune response was measured by serum ELISA.

EBNA1-UNITE vaccine prevents EBNA1 expressing CT26 colon tumor growth in BALB/c mice and generates Ag-specific memory response

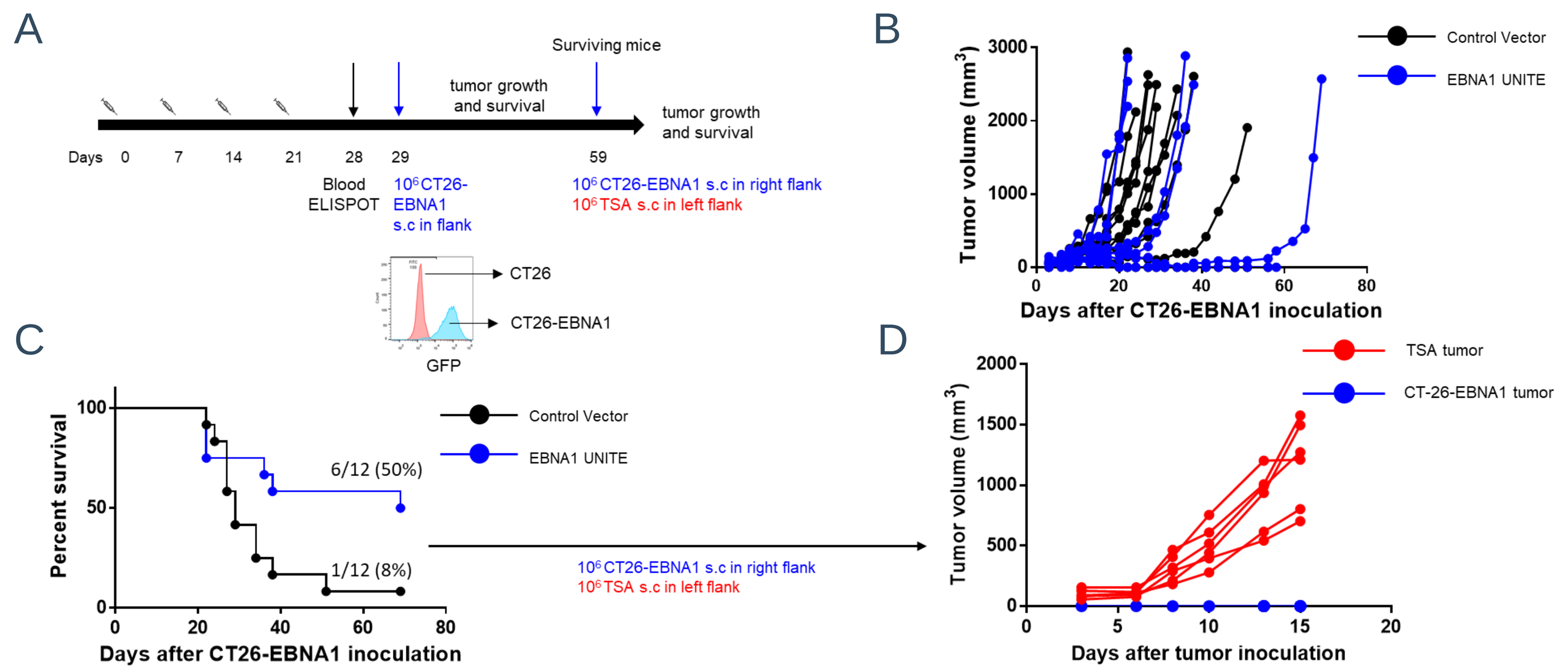


Figure 2: Study design. BALB/c mice were immunized with the vaccine by i.d. delivery in ear and electroporation on days 0, 7,14, and 21. Mice were bled on day 28 to assess immune response by ELISPOT. CT26 cells were transduced to express GFP-EBNA1, GFP expression was measured by flow cytometry (A). On day 29 vaccinated mice were injected with CT26-EBNA1. Tumor growth (B) and survival (C) were recorded. The surviving mice from C were re-injected with CT26-EBNA1 tumor in right flank and TSA tumor in left flank. TSA and CT26-EBNA1 tumor growth was recorded as in D.

EBNA1-UNITE vaccine increases infiltration of activated TNFα-producing $CD4^+$EMT and $CD8^+$EMT cells in the TME

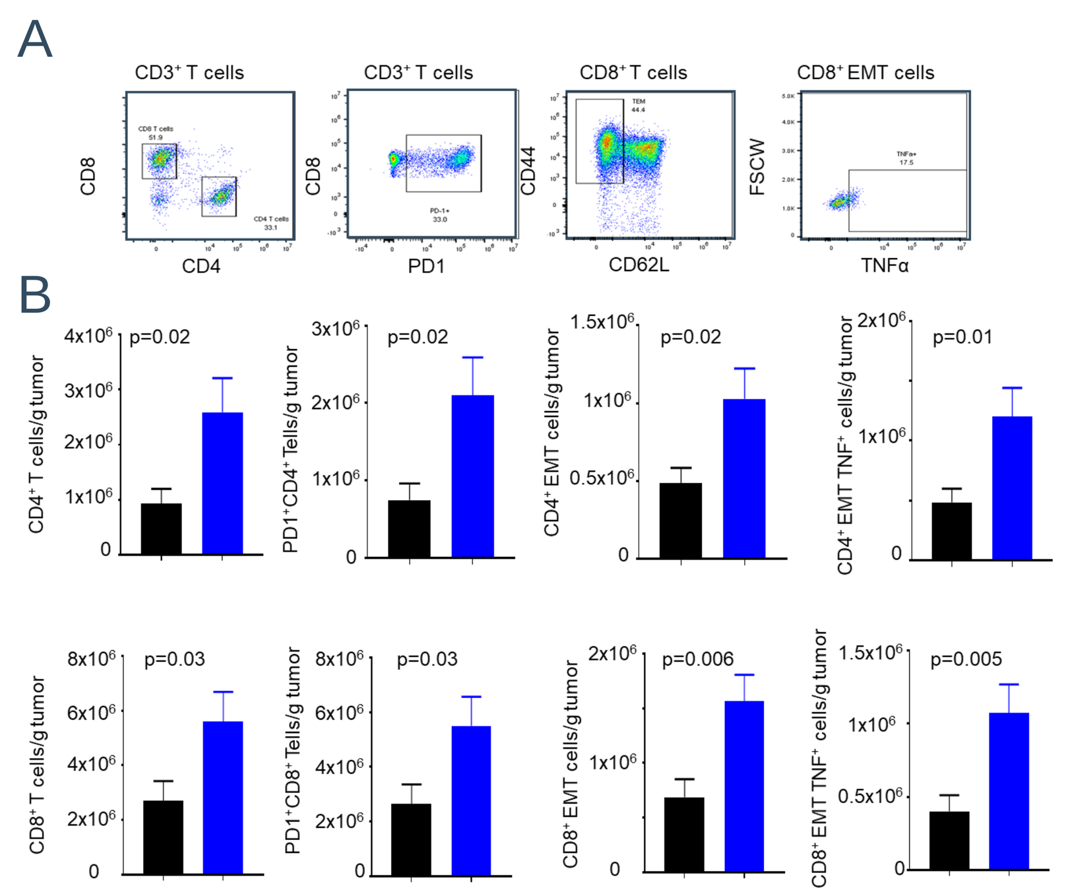


Figure 3: BALB/c mice were inoculated with 10^6 CT26-EBNA1 cells. Mice with $<100mm^3$ tumor volume were immunized with the vaccine or control vector on day 7 and day 14. Brefeldin A (BFA) 250ug per mouse in 200ul PBS was injected ip in each mouse on day 21 and the tumors were harvested 4h after BFA inoculation. Tumors were cleaned, weighed, digested using Miltenyi tumor dissociation kit and the gentleMACS dissociator, and stained with fluorescent antibodies. **A.** Gating scheme. **B.** Histogram of average + SEM cells from each group. Black = control vector, blue = EBNA1-UNITE

EBNA1-UNITE vaccine promotes tumor infiltration of activated NKT cells

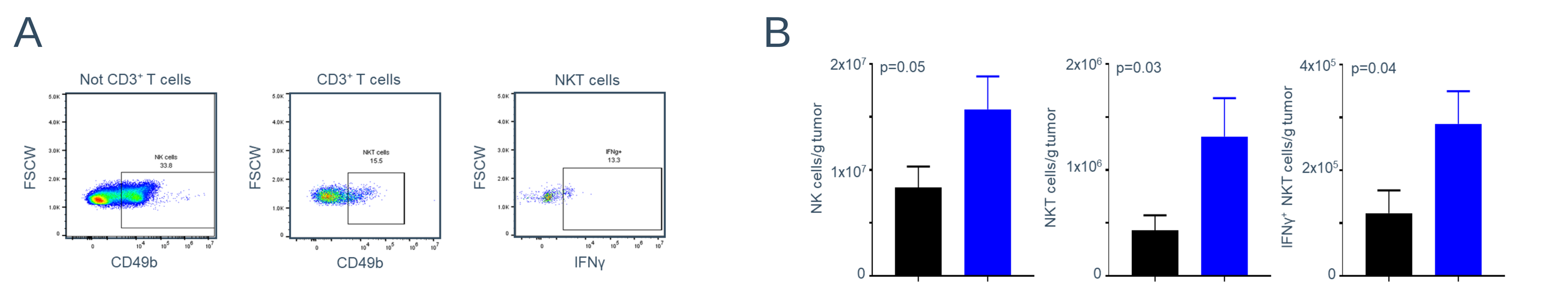


Figure 4: Method of tumor inoculation, vaccination, and tumor preparation was same as figure 3. Tumors were stained with fluorescent antibodies. A. Gating scheme. B. Histogram of average + SEM cells from each group. Black = control vector, blue = EBNA1-UNITE

EBNA1-UNITE vaccine induces IL-12-producing dendritic cells in the tumor microenvironment

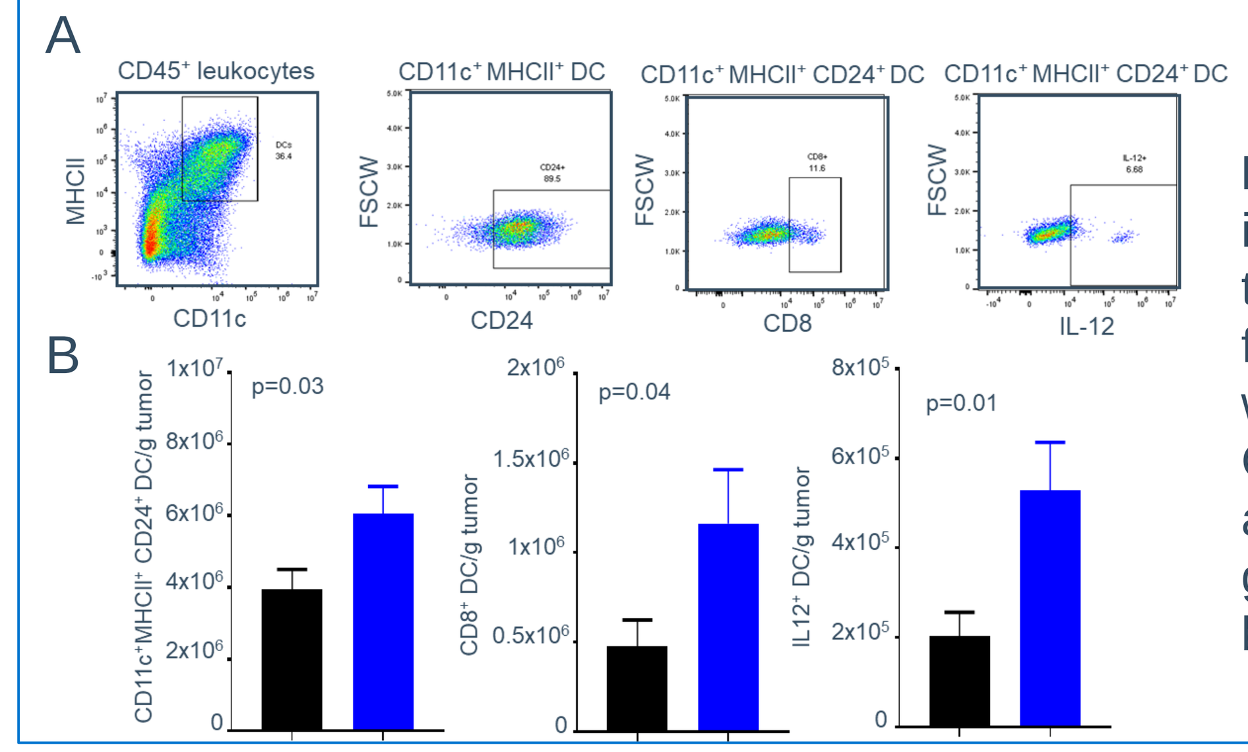


Figure 5: Method of tumor inoculation, vaccination, and tumor preparation was same as figure 3. Tumors were stained with fluorescent antibodies. A. Gating scheme. B. Histogram of average + SEM cells from each group. Black = control vector, blue = EBNA1-UNITE

Conclusions

- EBNA1-UNITE vaccine elicits robust EBNA1-specific $CD4^+$T and $CD8^+$T cell responses in vivo, which in turn have significant antitumor effect in EBNA1-expressing murine CT26 colon cancer model.
- Mice rejecting tumors were protected from re-challenge with CT26-EBNA1, demonstrating that antigen-specific memory was induced in these animals.
- EBNA1-UNITE vaccine significantly promotes tumor infiltration with activated EM $CD4^+$T and EM $CD8^+$T cells suggesting LAMP benefits both $CD4^+$T and $CD8^+$T cell infiltration.
- EBNA1-UNITE vaccine promotes tumor infiltration of activated NKT cells.
- EBNA1-UNITE vaccine induces IL-12-producing dendritic cells in the tumor microenvironment.

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