Combining an engineered costimulatory vaccine with NK cells induces an anti-tumor effect against murine neuroblastoma *in vitro* and after bone marrow transplant *in vivo*

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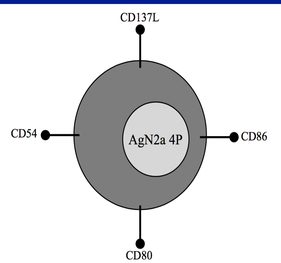
Abstract

High risk neuroblastoma remains a challenge to cure with only 50% survival, despite multi-modality treatment. Natural killer (NK) cells have been previously shown to have activity versus neuroblastoma but have not been consistently successful in clinical trials. NK cell activation via co-culture with a vaccine engineered to express CD54, CD80, CD86, and CD137L, called AgN2a 4P, was studied to investigate NK cells' ability to induce cytotoxicity of murine neuroblastoma tumor cells *in vitro* and *in vivo*. NKs and irradiated AgN2a 4P were co-cultured in ratios of 1 (NKs):0.5 (AgN2a 4P) and 1:1, and compared to NK only and AgN2a 4P only controls, with all groups receiving IL-15/IL-15Rα, and then analyzed by flow cytometry, multiplex cytokine analysis, and cytotoxicity *in vitro* after 1, 3, 5, 7, and 9 days. To study the efficacy of *in vivo* vaccination with AgN2a 4P after bone marrow transplant (BMT), C57BL/6 or B6AJ recipients were lethally irradiated, followed by transplantation of T-cell depleted C57BL/6 donor bone marrow on day +0. BMT recipients were then treated with the AgN2a 4P vaccine for 3 doses, and with or without adoptive transfer of donor NK cells to accelerate immune reconstitution. Our group has previously shown, *in vivo,* the administration of 3 doses of AgN2a 4P is most effective. All recipients were then challenged with Neuro2a or 9464D neuroblastoma tumor, and followed for tumor growth and survival. The NK:AgN2a 4P co-culture at 1:1 increases Ly49D+ NKs from 3% to 34% from day +0 to day +9. pSTAT1 activation remains consistently high between 80%-98% across the co-culture period. NK cells release increased levels of IFN-gamma and IL-6 at the co-cultured ratios of 1:0.5 and 1:1, and CXCL1 at the 1:1 ratio, as compared to the NK with IL-15/IL-15Rα controls. The NK:AgN2a 4P ratios of 1:0.5 and 1:1 induce significantly increased apoptosis of Neuro2a and 9464D neuroblastoma cells than NK cells with IL-15/IL-15Rα alone. *In vivo,* AgN2a 4P with adoptive transfer of donor NK cells induces an anti-tumor effect, slowing tumor growth considerably. Co-culture of NK cells with an engineered costimulatory vaccine is an effective strategy to induce apoptosis of neuroblastoma tumor cells by increasing NK-mediated cytokine production and cytotoxicity, and enhances anti-tumor effects after BMT. Usage of cell-based vaccines after BMT could be an effective strategy to augment NK cell activity against neuroblastoma.

Introduction

Figure 1. AgN2a 4P vaccine. AgN2a 4P is an aggressive variant of the murine Neuro-2a neuroblastoma cell line engineered by nucleofection to express:

CD54 (ICAM-1 adhesion molecule that binds LFA-1 and MAC-1 on T cells and NK cells)
CD80 (co-stimulatory molecule for T-cell activation)
CD86 (co-stimulatory molecule for T-cell activation)
CD137L (co-stimulatory molecule for T cell and NK cell activation)



Ref: Johnson BD et al. *J Immunother* 2005 Sep-Oct;28(5):449-60

- There are no current vaccines in the clinic that prevent recurrence of neuroblastoma.
- Currently, neuroblastoma treatment involves the use of only autologous bone marrow transplant.

In vitro Model of NK:AgN2a 4P Co-culture

Group 1 → (1:0.5) = 1x10^6 NK cells + 0.5x10^6 irradiated$^+$ AgN2a 4P cells

Group 2 → (1:1) = 1x10^6 NK cells + 1x10^6 irradiated$^+$ AgN2a 4P cells

Group 3 → (NK ONLY Control) = 1x10^6 NK cells

Group 4 → (AgN2a 4P ONLY Control) = 1x10^6 irradiated$^+$ AgN2a 4P cells

*Each group received 5mL of Complete Mouse Media (CMM) + IL-15/IL-15Rα (10ng/mL) every other day starting on day +0.

$^+$AgN2a 4P cells were irradiated at 100Gy.

Figure 2. Experimental design for *in vitro* co-culture of C57BL/6 NK cells with AgN2a 4P. NK cells were isolated from the spleen of C57BL/6 mice using magnetic cell selection Groups were tested on days 1, 3, 5, 7, and 9 utilizing flow cytometry, multiplex cytokine analysis, and cytotoxicity assays.

Methods

Figure 3. BMT model and AgN2a 4P vaccination schedule. Schedule is in accordance with groups in **Figure 4**.

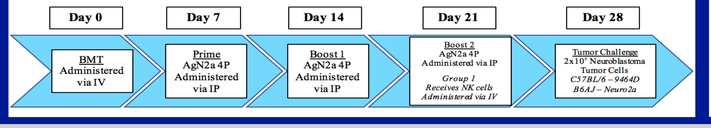
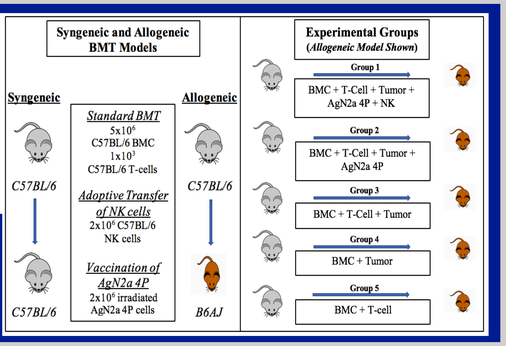


Figure 4. BMT schema and description of experimental groups. Syngeneic is an MHC matched donor; allogeneic is an MHC mismatched donor. All mice receiving BMT are lethally irradiated at 11Gy. C57BL/6 mice were used as donors.



Results

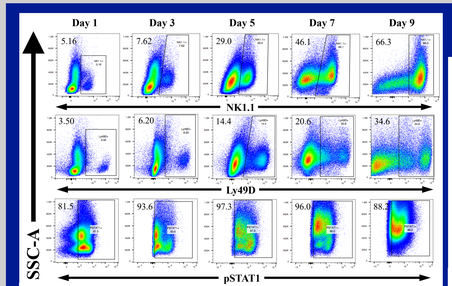


Figure 5. Flow cytometric analysis of C57BL/6 NK cells co-cultured with irradiated AgN2a 4P *in vitro* at a 1:1 ratio (NK:AgN2a 4P). Analysis of cell surface markers NK1.1 (top) and Ly49D (middle), and transcription factor pSTAT1 (bottom). An increase in NK1.1 and Ly49D occurs over the 9-day co-culture period, while pSTAT1 expression remains high at all time points (1, 3, 5, 7, and 9 days).

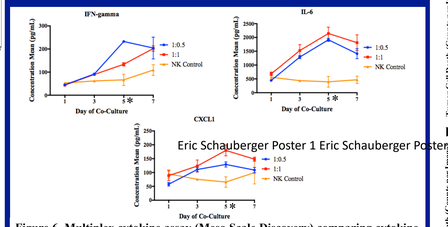


Figure 6. Multiplex cytokine assay (Meso Scale Discovery) comparing cytokine production between different *in vitro* co-culture ratios (NK:AgN2a 4P). The 1:1 NK:AgN2a 4P ratio, compared to the NK only control group, has a greater increase in cytokine production of gamma interferon (IFN-gamma), IL-6, and CXCL1.
*Day 5 has statistical significance between the different co-cultured ratios: IFN-gamma (p-value = 0.0094); IL-6 (p-value = 0.0175); CXCL1 (p-value = 0.0478).

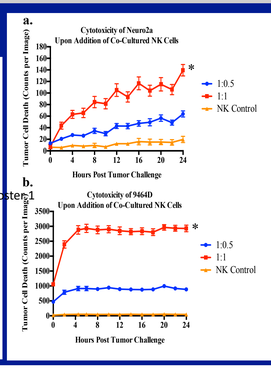


Figure 7. Cytotoxicity assay (Incucyte) of neuroblastoma tumor cells by addition of C57BL/6 NK cells co-cultured with AGN2a 4P and IL15/IL-15α (10ng/mL) at a 10:1 (effector:target) ratio. Effector NK cells are added after 7 days of co-culture with AgN2a 4P.
a. Target cells are Neuro-2a (allogeneic) murine neuroblastoma. The 1:1 (NK:AgN2a) ratio induces significantly higher cytotoxic activity *(p-value <0.0001) against Neuro-2a tumor cells than the 1:0.5 ratio and the NK only control.
b. Target cells are 9464D (syngeneic) murine neuroblastoma. The 1:1 (NK:AgN2a) ratio induces significantly higher cytotoxic activity *(p-value <0.0001) against 9464D tumor cells than the 1:0.5 ratio, and the NK only control.

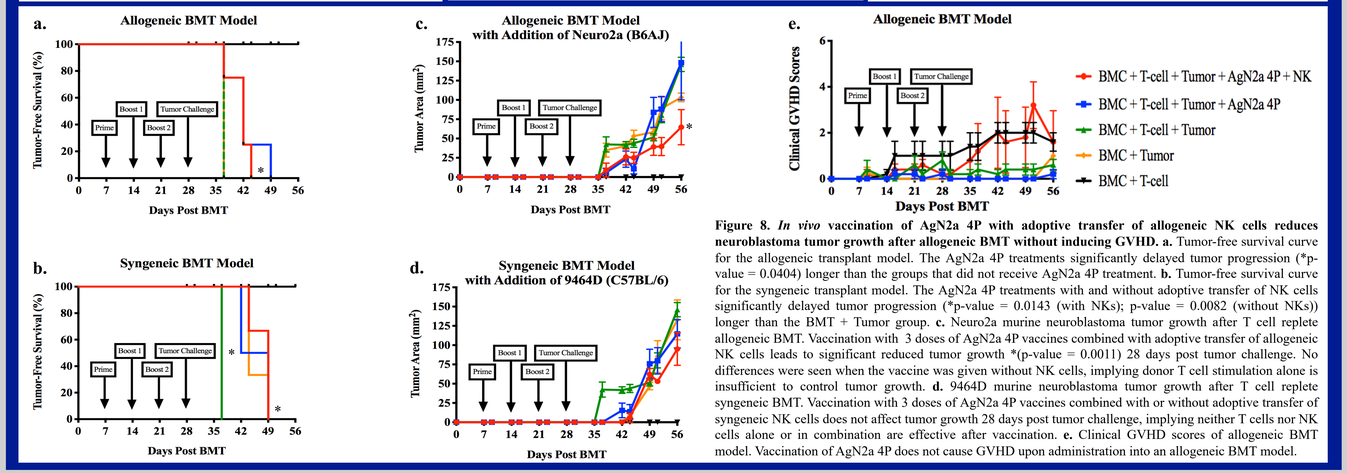


Figure 8. *In vivo* vaccination of AgN2a 4P with adoptive transfer of allogeneic NK cells reduces neuroblastoma tumor growth after allogeneic BMT without inducing GVHD. a. Tumor-free survival curve for the allogeneic transplant model. The AgN2a 4P treatments significantly delayed tumor progression (*p-value = 0.0404) longer than the groups that did not receive AgN2a 4P treatment. **b.** Tumor-free survival curve for the syngeneic transplant model. The AgN2a 4P treatments with and without adoptive transfer of NK cells significantly delayed tumor progression (*p-value = 0.0143 (with NKs); p-value = 0.0082 (without NKs)) longer than the BMT + Tumor group. **c.** Neuro2a murine neuroblastoma tumor growth after T cell replete allogeneic BMT. Vaccination with 3 doses of AgN2a 4P vaccines combined with adoptive transfer of allogeneic NK cells leads to significant reduced tumor growth *(p-value = 0.0011) 28 days post tumor challenge. No differences were seen when the vaccine was given without NK cells, implying donor T cell stimulation alone is insufficient to control tumor growth. **d.** 9464D murine neuroblastoma tumor growth after T cell replete syngeneic BMT. Vaccination with 3 doses of AgN2a 4P vaccines combined with or without adoptive transfer of syngeneic NK cells does not affect tumor growth 28 days post tumor challenge, implying neither T cells nor NK cells alone or in combination are effective after vaccination. **e.** Clinical GVHD scores of allogeneic BMT model. Vaccination of AgN2a 4P does not cause GVHD upon administration into an allogeneic BMT model.

Conclusion

- Co-culture of murine NK cells and AgN2a 4P cells in IL-15 increases the percentage of Ly49D$^+$ NK cells *in vitro,* as well as secretion of higher concentrations of IFN-gamma, IL-6, and CXCL1, as compared to IL-15 activated NK cells alone.
- Murine NK cells co-cultured with AgN2a 4P and IL-15 induce higher levels of cytotoxicity to two separate murine neuroblastoma tumors, compared to IL-15 activated NK cells alone. The 1:1 ratio of NK:AgN2a 4P is the most effective cytotoxic ratio tested.
- After T cell replete allogeneic BMT, combining the AgN2a 4P vaccine with NK cells significantly reduces tumor growth as compared to BMT + vaccination or BMT alone, implying donor T and NK cells are needed for vaccine effectiveness.
- AgN2a 4P is a safe and potentially effective vaccine for stimulating NK cells *in vitro* and enhancing a T cell and NK cell-mediated graft-versus-tumor effect *in vivo*.

Acknowledgements

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