

# Modulated electro-hyperthermia and combined primary, immortalized NK-cell therapy in human A2058 xenograft model

Tamas Vancsik<sup>1,2</sup>, Domokos Mathe<sup>3</sup>, Anett Benedek<sup>2</sup>, Ildiko Horvath<sup>2</sup>, Nikolett Hegedus<sup>3</sup>, Ralf Bergmann<sup>3</sup>, Csaba Schvarcz<sup>2</sup>, Erno Papanek<sup>2</sup>, Tibor Krenacs<sup>1</sup>, Zoltan Benyo<sup>2</sup>, Andrea Balogh<sup>2</sup>

<sup>1</sup>1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary

<sup>2</sup>Institute of Clinical Experimental Research, Semmelweis University, Budapest, Hungary

<sup>3</sup>Department of Biophysics and Radiation Biology Semmelweis University, Budapest, Hungary

**Citation:** Vancsik T. et al. (2020): Modulated electro-hyperthermia and combined primary, immortalized NK-cell therapy in human A2058 xenograft model. *Oncothermia Journal* 29: 135 – 144, [www.oncotherm.com/sites/oncotherm/files/2021-02/Vancsik\\_ImmortalizedNKcell](http://www.oncotherm.com/sites/oncotherm/files/2021-02/Vancsik_ImmortalizedNKcell)

## Abstract

**Introduction:** Earlier we showed that modulated electro-hyperthermia (mEHT) promoted the expression and release of the potential immunogenic damage associated molecular pattern proteins and it reduced MHC-I and melan-A levels in B16F10 melanoma cells. The number of cytotoxic T cells were moderately reduced, the amount of NK cells was unchanged. NK cells could effectively recognize and kill cells which lack MHC-I. Here we tested the effect mEHT on tumor growth and tumor microenvironment with respect to infiltration and cytotoxicity of NK cells in A2058 human melanoma xenograft model *in vivo*.

**Material and methods:** A2058 melanoma cells were inoculated into both flanks of BALB/C NOD/SCID immunocompromised mice. After two weeks, 30-min 42°C mEHT was applied on the right-side tumors. One day after mEHT treatment, primary human NK-cells or the NK92MI NK-cell line labeled with fluorescent dye were injected subcutaneously above the lumbar region of the spine. NK-cell distribution was measured by *in vivo* fluorescent imaging. Tumor size was monitored using ultrasonic caliper. Tumor damage, growth arrest, heat stress and apoptosis related markers were assessed with immunohistochemistry. NK-attracting CXCL mRNA expression was determined after *in vitro* mEHT treatment of A2058 cells.

**Results:** mEHT induced significant tumor growth inhibition. Heatshock and apoptotic tumor cell death was proven by the significant elevation of relative dead tumor area,  $\gamma$ H2AX, p53 and cleaved caspase-3 and hsp70 positive areas, accompanied by MMP-2 expression. *In vivo*, both the primary NK- and NK92MI-cells accumulated into the mEHT-treated side and further enhanced the damaging effect. Significant elevation of CXCL-11 mRNA level, was induced by *in vitro* treatment while the CXCL-9, and -10 dropped.

**Conclusion:** Our result show that mEHT can induce p53-mediated caspase-dependent apoptosis in an A2058 melanoma xenograft model. Furthermore, mEHT treatment may provide a favorable micro-environment for the attraction and invasion of NK-cells, possibly by inducing CXCL-11 expression and promoting MMP-2 production of solid xenografts.

This study was funded by NKFIH-NVKP\_16-1-2016-0042.

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## Introduction I.

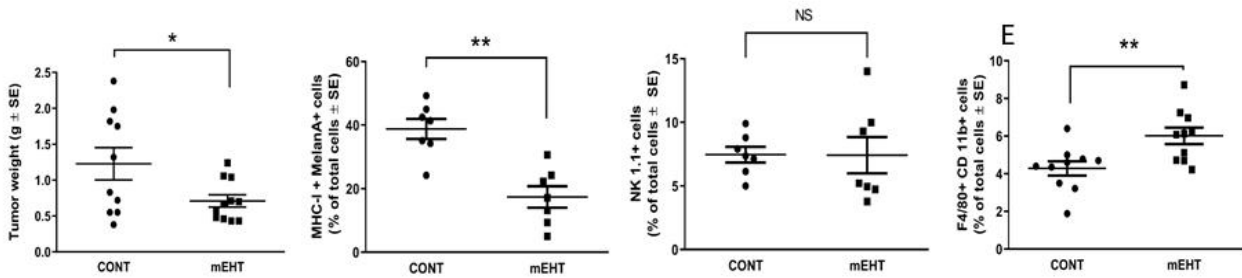
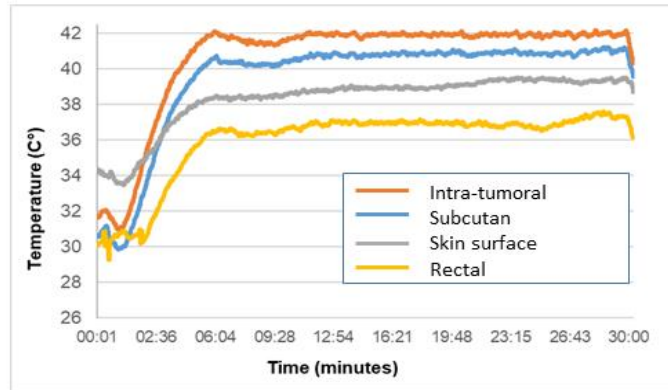
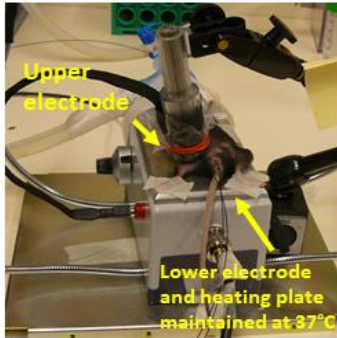
### Melanoma

- SEER: poor (16.2%) five-year survival rate of melanoma patients with regional/distant spreading.
- Effectively suppressed adaptive anti-tumor immune response by the tumor cells.
- Potential targets:
  - PD-L1 and CTLA-4 checkpoint proteins produced by tumor cells, may induce programmed cell death of the effector CD8+ cytotoxic T-cells.
- mEHT as a complementary:
  - tumor-damaging effects by irreversible heat and cell stress
  - tolerable for patients, with almost no side-effects.
  - Upregulation and release of damage-associated molecular pattern (DAMP) proteins, which are accompanied by progressive immune-mediated secondary tumor-damage (immunogenic cell death)\*

\*Vancsik, T.; Kovago, C.; Kiss, E.; Papp, E.; Forika, G.; Benyo, Z.; Meggyeshazi, N.; Krenacs, T. **Modulated electro-hyperthermia induced loco-regional and systemic tumor destruction in colorectal cancer allografts.** J. Cancer 2018, 9, 41–53.

## Introduction II.

### Mouse B16F10 melanoma allografts in immunocompetent C57Bl/6 mouse model



Besztercei B<sup>1</sup>, Vancsik T<sup>2</sup>, Benedek A<sup>1</sup>, Major E<sup>1</sup>, Thomas MJ<sup>1</sup>, Schvarcz CA<sup>1</sup>, Krenács T<sup>2</sup>, Benyó Z<sup>1</sup>, Balogh A<sup>3</sup>. **Stress-Induced, p53-Mediated Tumor Growth Inhibition of Melanoma by Modulated Electrohyperthermia in Mouse Models without Major Immunogenic Effects.** *Int J Mol Sci.* 2019 Aug 17;20(16). pii: E4019. doi: 10.3390/ijms20164019.

## Aims of study

### Human A2058 melanoma xenografts in immunodeprived NOD SCID mouse model

#### Hypothesis

mEHT can reduce MHC-I on tumor cell surface and induce tumor cell stress, thus can activate the natural killer cells to eradicate the malignant cells.

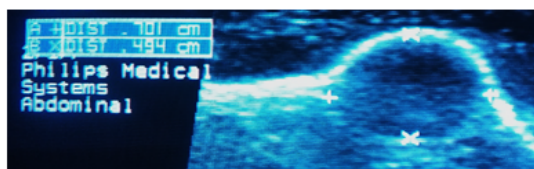
#### Model

- A2058 human melanoma cell line; functional p53
- NOD/SCID mice: deficient in T, B, NK cells and complement system
- 30 min of 42°C mEHT
- subcutan injected fl. labelled human NK cells

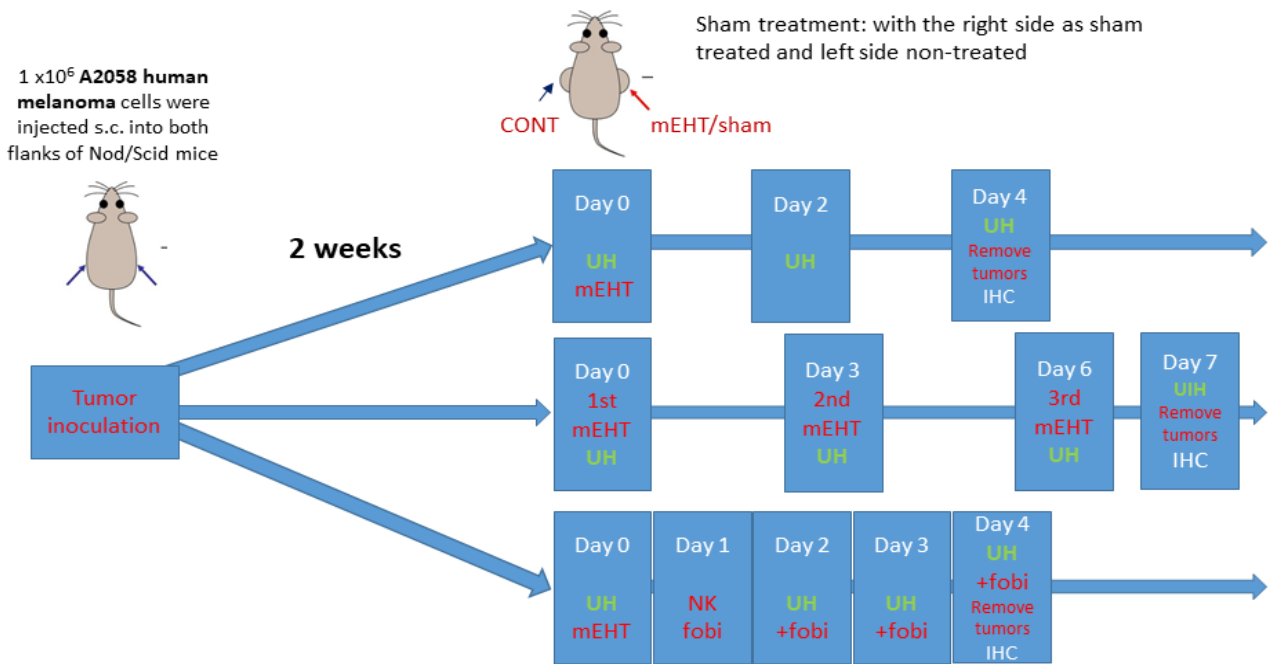
#### Analysis

- tumor growth monitoring with UH measurement
- in vivo imaging of NK distribution with FOBI detection system
- IHC: cytochrome-c, caspase-3, AIF, HSP70, p53, p21, H2AX, F4/80

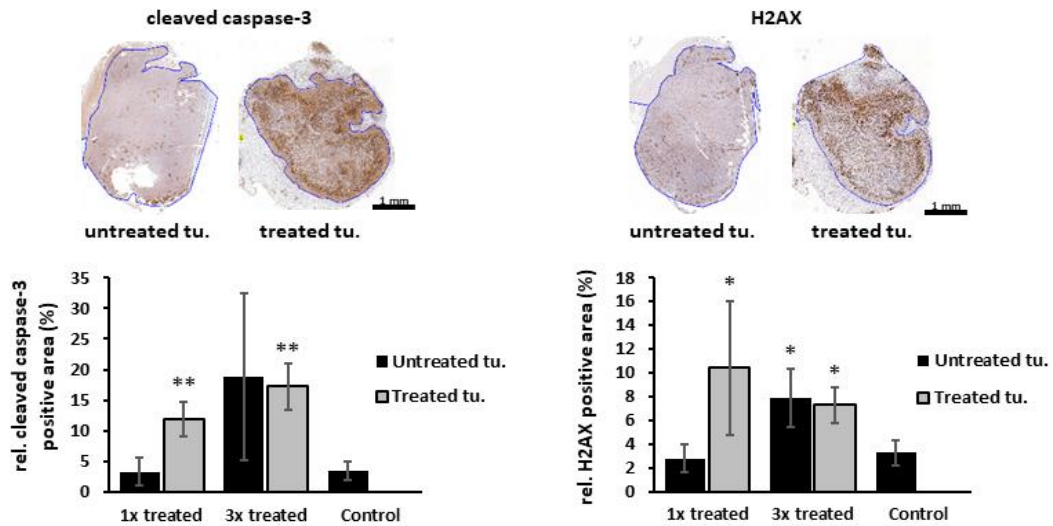
*A2058 melanoma xenografts in NOD SCID mouse*



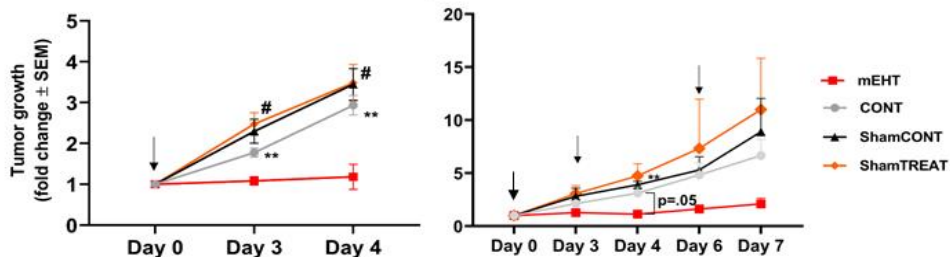
## Experimental protocols: treatment is done one or multiple times



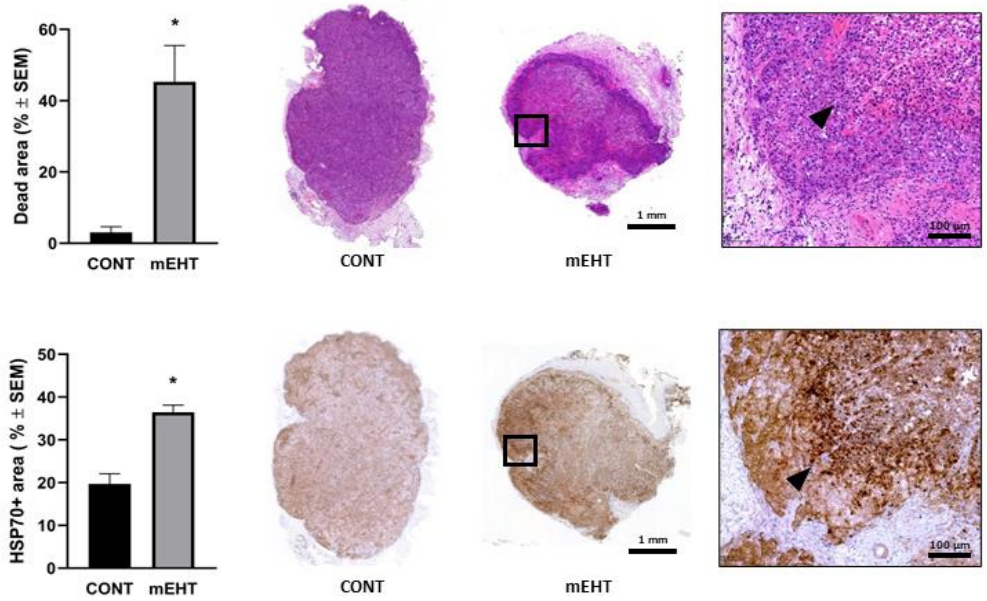
## IHC markers analysed so far in the 1 x and 3 x treated tumors



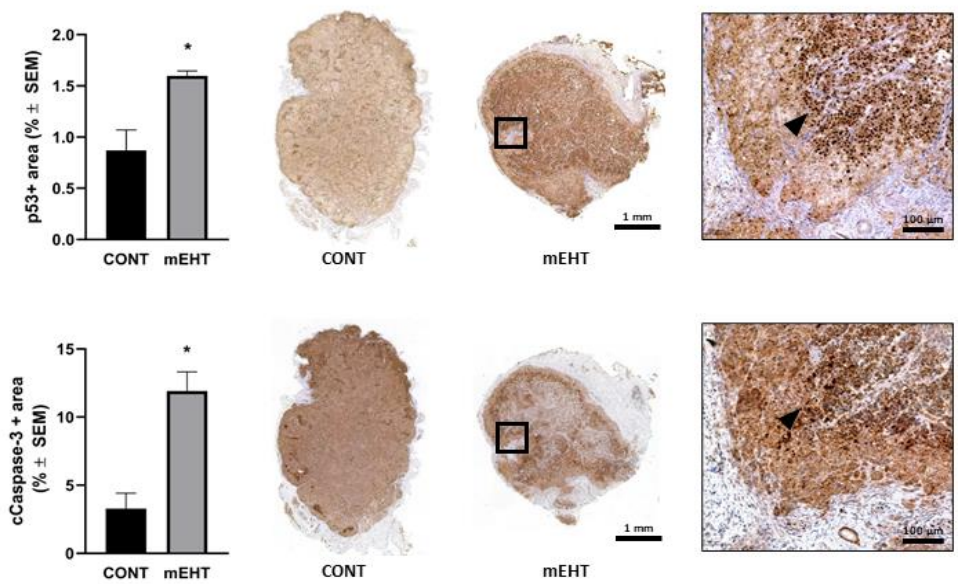
## Tumor growth



### Tumor damage and heat stress

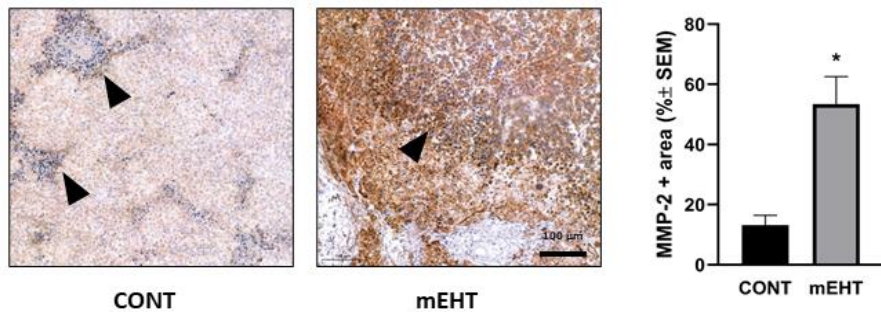


### p53 dependant apoptosis

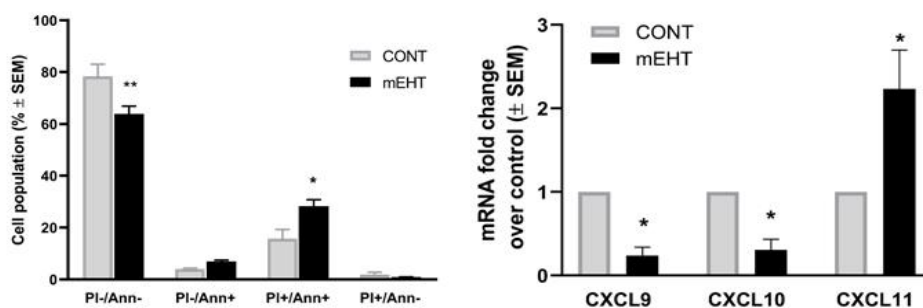


## Changes in the tumor microenvironment

matrix metalloprotease-2 expression *in vivo*



tumor cell apoptosis and NK-attractive cytokine mRNA expression *in vitro*



## NK-cell therapy

Source of human NK cells:

- Isolation and expansion of primary human NK cells (CD56, granzyme expression)
- Commercially available NK92mi cell line

Functional analysis:

- *in vitro* kill of A2058 melanoma cells

Subcutan injection to lumbar region

Detect their localization:

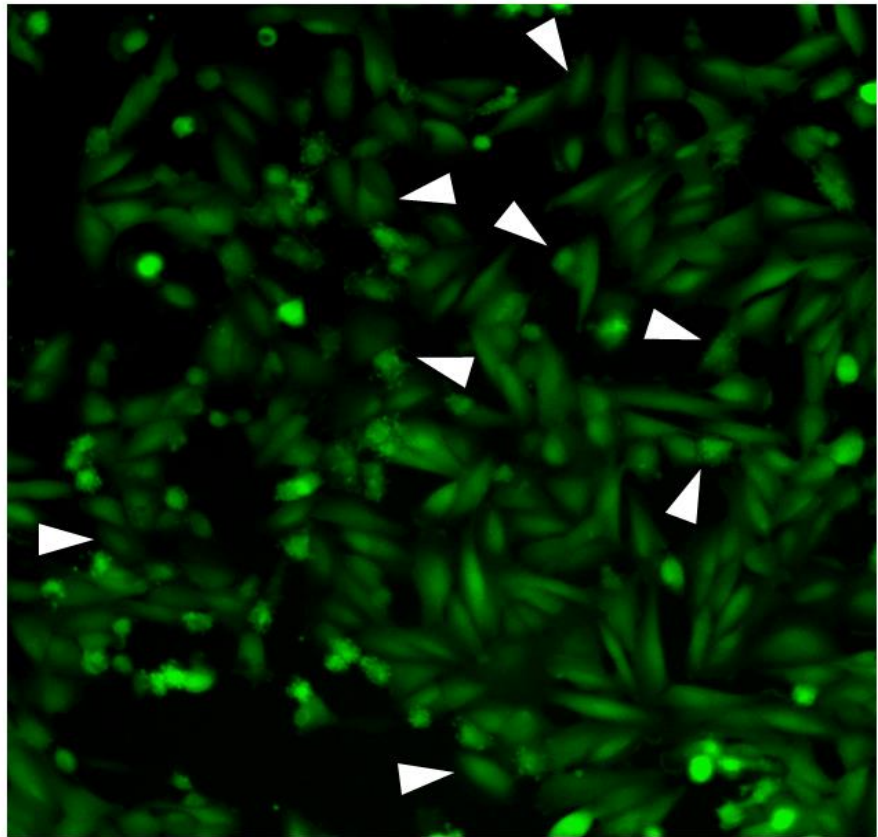
- NK cells were labelled with far red fluorescent dye and using *in vivo* imaging\*



\*FOBI *in vivo* detection system

## In vitro cytotoxicity of primary NK cells: life cell imaging

Calcein loaded  
A2058 cell culture  
In a 12 well plate  
 $15 \cdot 10^4$  NK-cells/well  
35 minutes  
1 picture/20 sec



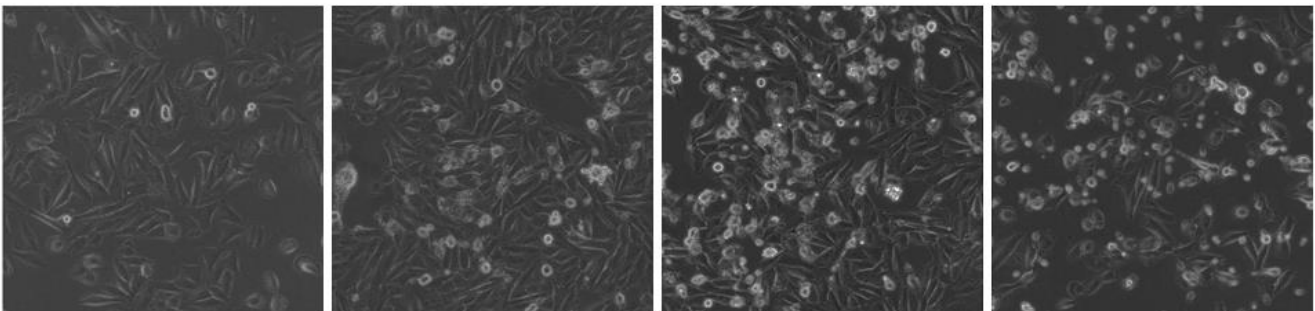
## In vitro cytotoxicity: microscopy

0 NK

$5 \cdot 10^4$  NK-cells/well

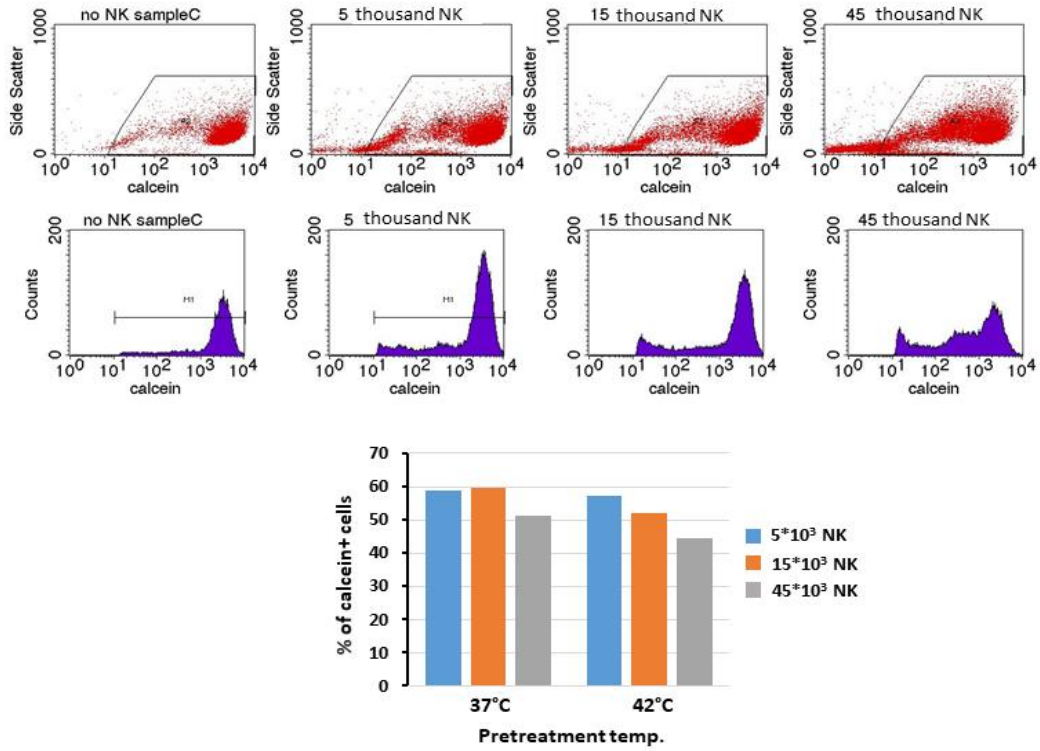
$15 \cdot 10^4$  NK-cells/well

$45 \cdot 10^4$  NK-cells/well

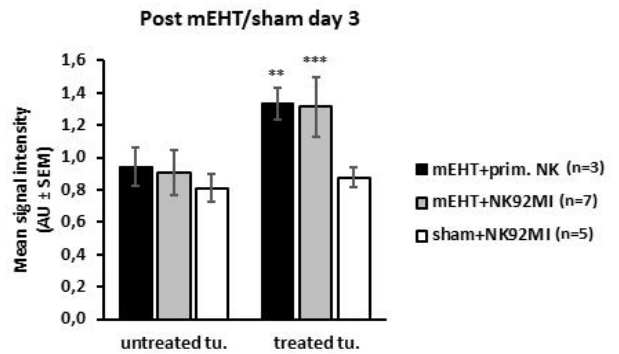
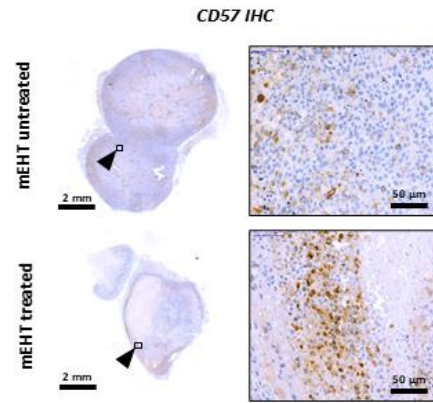
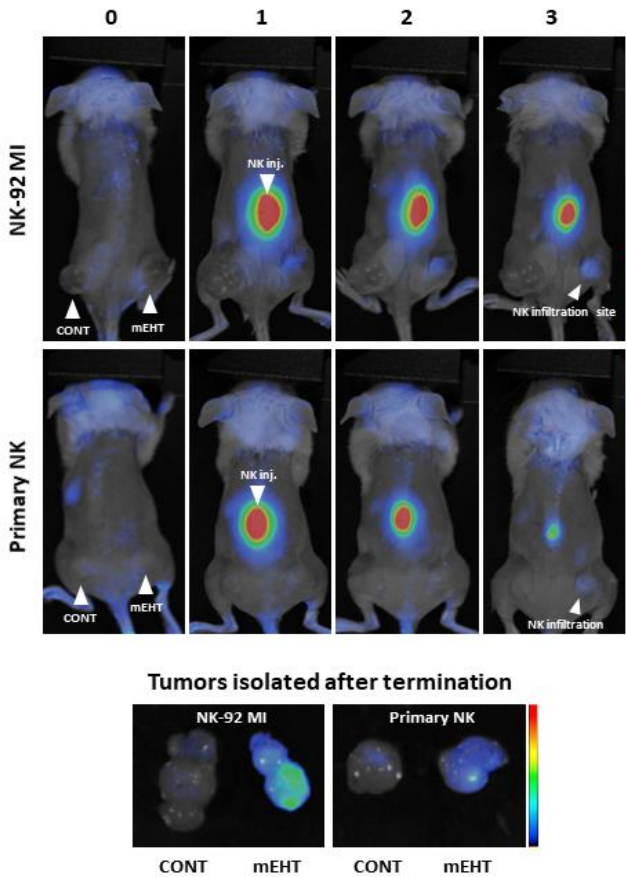


~2 hrs co-culturing

### Primary NK *in vitro* cytotoxicity: calcein release assay

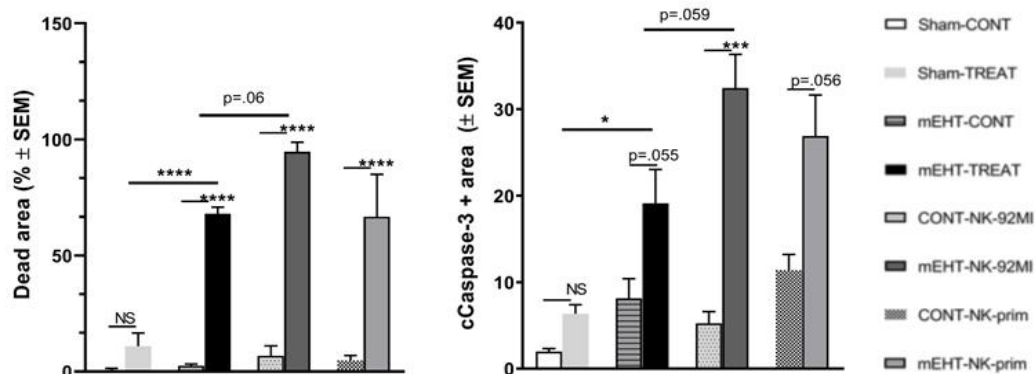
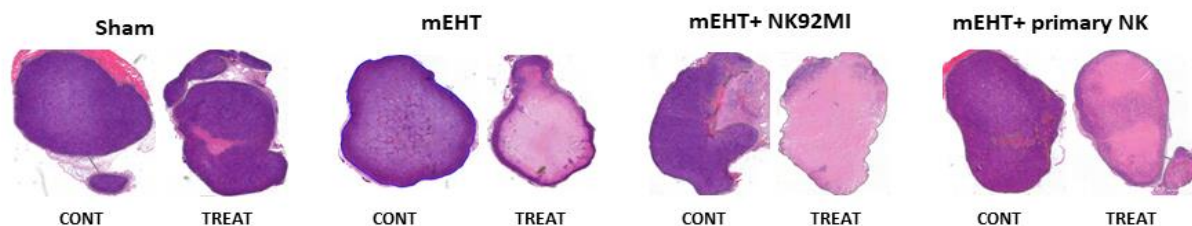


### Days relative to mEHT treatment and NK cell injection



0.01 > p > 0.001  
0.001 > p > 0.001

## Apoptotic area in context of NK-cell therapy



p>0.05: n=3;  
further elements are under  
evaluation

## Discussion

In a A2058 Xenograft model:

### **mEHT-related effects:**

- p53 activation → tumor growth inhibition
- DNA DSB and caspase-dependent apoptosis
- favorable microenvironment attracting NK cell trafficking

*In vitro* NK cell tumor cytotoxicity, and its accumulation on the mEHT treated tumor sites suggests the involvement of NK cells in A2058 melanoma cell killing

Further testing for the NK-attractive and -inductive factors is needed