Ex vivo expanded NK cells show potent anti-tumour activity against melanoma using a combination of multiple activating mechanisms

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Key findings

- GTA002 exert efficient anti-tumour cytotoxicity against melanoma cell lines *in vitro*
  - As confirmed by degranulation, increase in intracellular levels of IFNγ and TNFα and release of perforin and granzyme B
  - Cytotoxic capacity strongly correlates with perforin and granzyme B levels in GTA002 before co-culture
- GTA002 cytotoxicity against melanoma is dependent on a combination of different activating receptors
  - Single receptor blocking of DNAM-1, NKG2D, NKp30, NKp44 or NKp46 resulted in minor reduction of cytotoxicity
  - GTA002 cytotoxicity against melanoma is highly dependent on the TRAIL pathway
  - Combining blockage of different activating receptors completely abrogated the cytotoxicity but is highly dependent on the target cell line

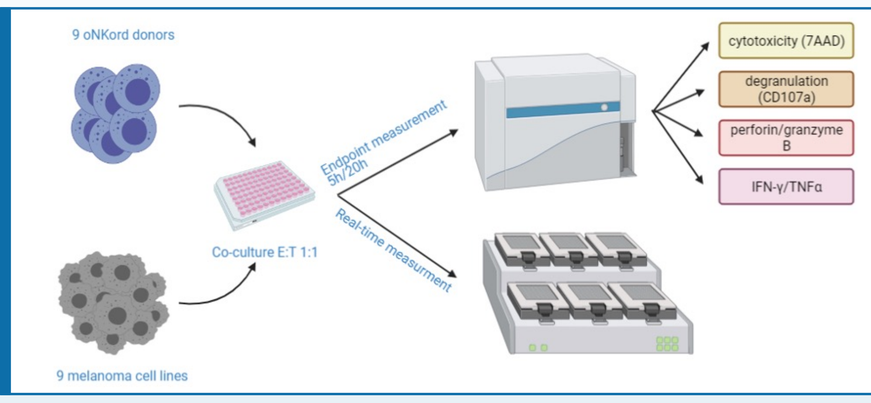
Method

Cytotoxicity screening: The cytotoxicity of 9 thawed GTA002 donors was determined against 9 different melanoma cell lines. Read-outs were based on target cell death detection and the intracellular measurement of important pro-inflammatory cytokines, such as IFNγ and TNFα, and the cytotoxic effector molecules perforin and granzyme B.
Cytotoxic activation mechanisms: Different receptor-ligand interactions were interfered with using blocking antibodies directed against specific NK cell receptors, alone or in combination, and the effect on cytotoxicity was measured using an impedance-based system.

Background

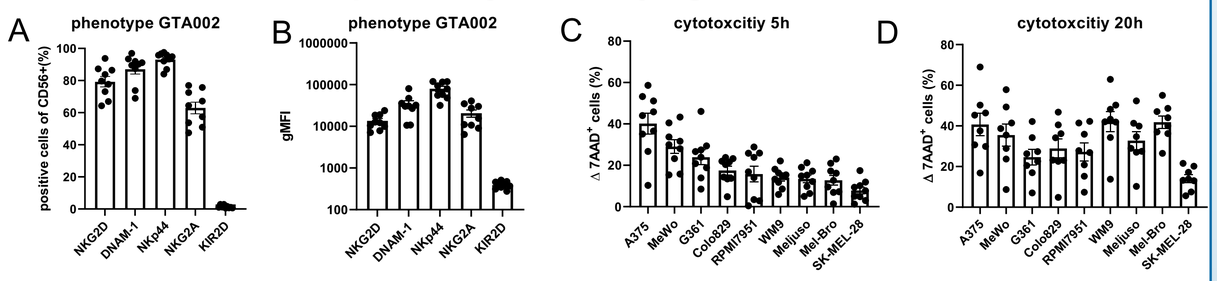
Glycostem Therapeutics (Oss, the Netherlands) developed a platform to expand and differentiate CD34$^+$ hematopoietic stem cells from umbilical cord blood into highly active NK cells, GTA002 (*Spanholtz et al., 2011*). These NK cell can target tumour cells without prior sensitization and are active against different types of tumours.
We investigated whether melanoma is a good target for our *ex vivo* generated NK cell product GTA002 and explored the cytotoxic mechanisms against melanoma after cryopreservation.

Experimental set-up



Results

1. GTA002 exert potent cytotoxicity against melanoma cell lines



*(**A&B**) GTA002 receptor expression in percentage and MFI. (**C&D**) Flow-based cytotoxicity results against 9 melanoma cell lines of a (**C**) 5h co-culture and (**D**) 20h co-culture assay, at E:T ratio of 1:1. The order of the cell lines in the graphs is arranged from high susceptibility to low susceptibility to GTA002 in the 5-hour co-culture. Each data point represents an individual donor and data are shown as mean ± SEM (n=8-9).*

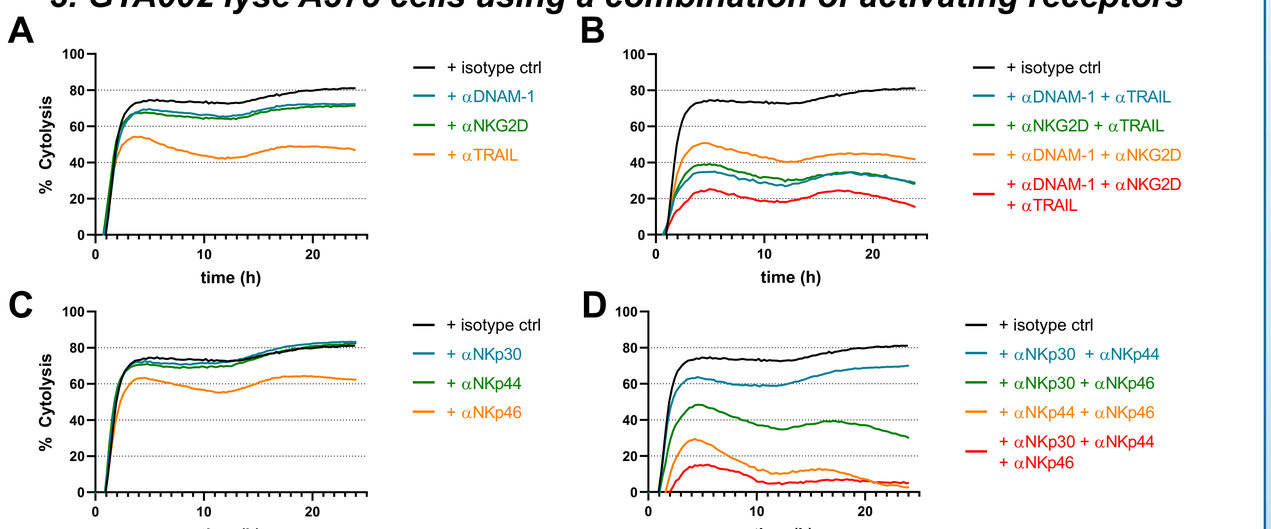
Terminally differentiated GTA002 express high levels of activating receptors NKG2D, DNAM-1 and NKp44, high expression levels of the inhibiting receptor NKG2A and low expression levels of KIR2D. Strong cytotoxic responses against 9 different melanoma cell lines were observed at low E:T ratio already after 5h and where even more pronounced at 20h.

2. GTA002 exposure to melanoma induces pro-inflammatory cytokine and cytotoxic effector molecule responses and correlates with cytotoxicity

*(**A-F**) GTA002 cells were co-cultured with melanoma cell lines for 5h, E:T ratio of 1:1 and percentage of (**A**) cytotoxicity, (**B**) degranulation, (**C**) IFNγ$^+$ cells, (**D**) TNFα$^+$ cells and gMFI of (**E**) perforin and (**F**) granzyme B was determined. Cell lines are arranged in order of GTA002 cytotoxic susceptibility. The red dashed line indicates baseline levels of effector only. Each data point represents an individual donor and data are shown as mean ± SEM (n=9). Below each bar graph, the corresponding heatmaps of the 9 individual GTA002 NK donors are shown. Donors are ordered from moderate cytotoxic capacity to high cytotoxic capacity. (**G**) Pearson correlation analysis of % cytotoxicity vs. % degranulation, % IFNγ$^+$ cells, % TNFα$^+$ cells, gMFI of perforin or gMFI of granzyme B of effector + target (NK+T) conditions, per cell line. (**H**) Correlation analysis of % cytotoxicity vs. intrinsic levels of effector only (NK) of perforin or granzyme B, per cell line and gMean of all cell lines.*

Co-culture of GTA002 with melanoma cell lines induced degranulation and initiated a pro-inflammatory response characterized by increased intracellular levels of IFNγ and TNFα. Target exposure led to release of perforin and granzyme B. Overall, these surrogate markers for cytotoxicity (B-F) did not correlate with the differences in target susceptibility as the cytotoxic levels do not follow the same trend (A). However, the individual cytotoxic capacity of each GTA002 donor correlates with levels of IFNγ, perforin and granzyme B after co-culture. Moreover, intrinsic levels of perforin and granzyme B of GTA002 donors before co-culture strongly correlate with their cytotoxicity against most melanoma cell lines.

3. GTA002 lyse A375 cells using a combination of activating receptors

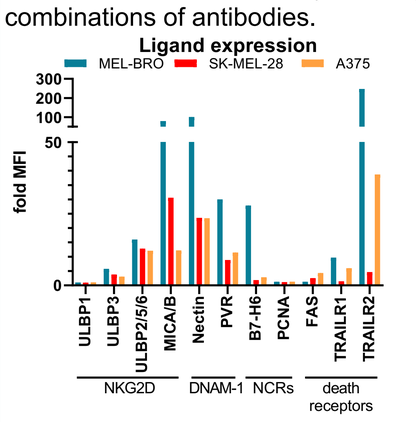


*GTA002 cells were pre-incubated with different combinations of blocking antibodies and seeded in co-culture with A375 in a 1:1 E:T ratio for 24h. Representative graphs of A375 cytolysis in co-culture with donor # 9 are shown as mean of technical triplicates after blocking (**A**) DNAM-1, NKG2D or TRAIL, (**B**) combination of DNAM-1, NKG2D and/or TRAIL, (**C**) NKp30, NKp44 or NKp46, (**D**) combination of NKp30, NKp44 and NKp46, (**E**) combination of NKp30 or NKp44 or NKp44 and TRAIL, (**F**) combination of NKp30, NKp44, NKp46 and TRAIL.*

Blocking single activating receptors such as DNAM-1, NKG2D, NKp30 or NKp44 did not affect cytotoxicity of GTA002 against A375, while blocking TRAIL or NKp46 reduced cytotoxicity, suggesting a relevant role for those activating receptors. However, combinations of blocking antibodies resulted in a cumulative effect of reduced cytotoxicity, showing involvement of all receptors in lysis of A375. Blocking TRAIL and an additional receptor led to greater inhibition of cytotoxicity, implying an important role for TRAIL in the function of GTA002.

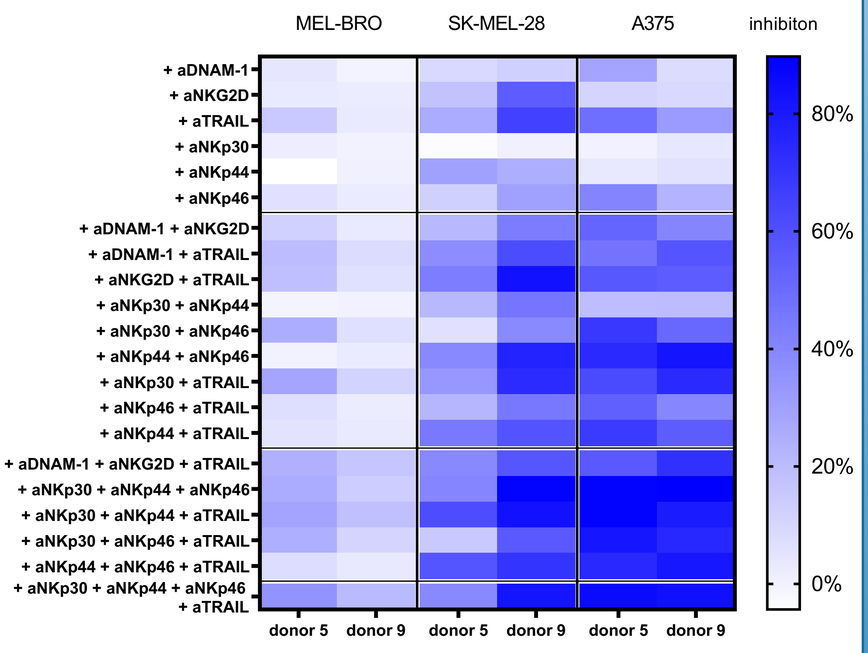
4. Target-dependent activation of GTA002 cytotoxic function

Similar to A375, the inhibition of cytotoxicity against SK-MEL28 requires a combination of multiple blocking antibodies. The trend of cytotoxicity inhibition between GTA002 donors was similar, implying that GTA002 becomes activated with similar mechanisms and their activation is target dependent. MEL-BRO is highly susceptible for GTA002 cytotoxicity, and it was very difficult to inhibit cytotoxicity with various blocking combinations of antibodies.



The fold MFI compared to unstained for ligand expression of MEL-BRO, SK-MEL-28 and A375.

A375, MEL-BRO and SK-MEL-28 cell lines showed substantial expression of ligands for NKG2D, DNAM-1, NCRs and death receptors. This enabled blocking studies to identify the specific receptor involvement in the mechanism of action (MoA) of GTA002 against melanoma.



*Heatmap of results of blocking studies. The percentage of cytotoxicity inhibition for each blocking combination is shown for MEL-BRO, SK-MEL-28 and A375 for two GTA002 donors. Percentage of cytotoxicity inhibition was calculated as 100-(AUC condition/ AUC isotype ctrl)*100. AUC= area under the curve.*

Future steps

The ability to inhibit cytotoxicity of GTA002 by different combinations of blocking antibodies against activating receptors suggests the involvement of these receptor-ligand interactions is important for GTA002 function. However, the differences in cytotoxic inhibition against cell lines suggests the potential involvement of differential inhibitory signalling. Clinical trials with GTA002 could generate data to link GTA002 MoA to clinical outcome and help define a predictive biomarker for the treatment of melanoma with GTA002.

PhD Student with expertise in primary human stem to NK cell expansion/differentiation & scientific focus on deciphering NK cell immunobiology through functional mechanistic approaches

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