Preclinical Characterization of FLEX-NK™ Tetravalent NKp46 Engager Directed Against GPC3 (CYT-303) Alone or in Combination With iPSC Derived Natural Killer Cells (iNKs) Against Hepatocellular Carcinoma (HCC)

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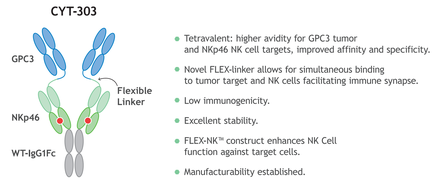
Cytovia Therapeutics

ABSTRACT

Glypican-3 (GPC3) is highly expressed in multiple solid tumors including HCC while it is hardly expressed in adult normal tissues except placenta. GPC3 promotes Wnt-dependent cell proliferation, and its expression is correlated with poor prognosis in HCC. NK cells exhibit innate anti-tumor activity owing to the expression of multiple activating receptors, such as NKp46. NKp46 is expressed in all NK cells including tumor-infiltrating NK cells. FLEX-NK™ is a proprietary platform for production of tetravalent IgG1-like multifunctional antibody NK engagers with a novel FLEX-linker to allow for simultaneous binding of both the targeted cancer cells and NK cells. A novel humanized NKp46 binder that does not induce NKp46 internalization and a humanized GPC3 binder that targets the membrane-proximal lobe of GPC3 were combined on the novel FLEX-NK™ scaffold to create the NK engager CYT-303. CYT-303 showed significantly higher dose dependent peripheral blood NK cell redirected cytotoxicity and degranulation against GPC3 expressing Hep3B tumor cells compared to GPC3 or NKp46 monoclonal antibodies alone suggesting that co-engagement of NKp46 and GPC3 via an immunological synapse is required for optimal tumor killing by CYT-303. CYT-303 treatment in PBNK (Peripheral Blood NK cells) injected NSG-IL15 mice showed greater Hep3B tumor growth inhibition compared to an IgG1 isotype control. Low NK cell numbers or suppression of NK cell function in the tumor microenvironment may limit the clinical activity of FLEX-NK™ engagers.

iNK cells derived from iPSCs, a uniform starting material with unlimited self-renewal capabilities, can be expanded to produce a universal off-the-shelf allogeneic therapy that can be used in combination with FLEX-NK™ engagers. We studied the efficacy of the combination of a FLEX-NK™ antibody and iNKs. The iNK cells express high levels of multiple activation receptors including NKp46 and showed good cytotoxic activity against the HCC cell line Hep3B. iNKs also showed anti-tumor activity in NSG-hIL15 mice bearing HCC subcutaneous tumors following intratumoral injection. CYT-303 greatly enhanced the cytotoxic activity of iNKs and cytolysis of Hep3B tumor cells *in vitro*. In a Hep3B tumor model in NSG-hIL-15 mice, the combination of CYT-303 and iNKs showed significantly greater tumor growth inhibition compared to iNKs alone plus an hIgG1 isotype control. Blood alpha fetoprotein (AFP) levels decreased in the CYT-303 plus iNK combination compared to iNK plus hIgG1 group. Cytokine release assessment of CYT-303 in the human PBMC assay showed no evidence of cytokine release while high levels of cytokine release was observed with anti-CD28 (TGN1412) and CD3 antibody controls. CYT-303 and iNK cells, alone or in combination, demonstrate anti-tumor activity against HCC that warrants clinical development.

FIGURE 1: FLEX-NK™ multifunctional engager in combination with iNK cells against HCC



- Tetravalent: higher avidity for GPC3 tumor and NKp46 NK cell targets, improved affinity and specificity.
- Novel FLEX-linker allows for simultaneous binding to tumor target and NK cells facilitating immune synapse.
- Low immunogenicity.
- Excellent stability.
- FLEX-NK™ construct enhances NK Cell function against target cells.
- Manufacturability established.

FLEX-NK™ multifunctional engager in combination with iNK cells against HCC

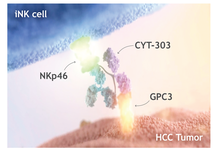
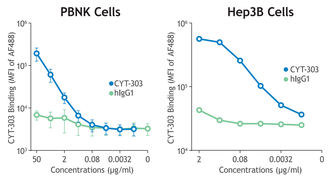
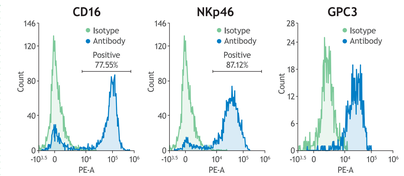


FIGURE 2: CYT-303 binding to NKp46 expressing peripheral blood NK cells and GPC3 expressing Hep3B tumors

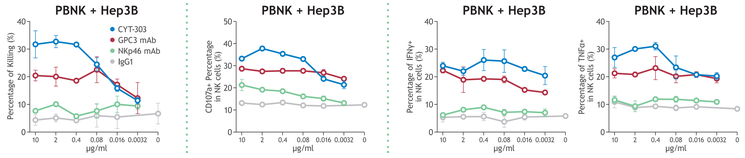


CYT-303 shows dose dependent binding to purified PBNK cells expressing NKp46 and Hep3B tumor cells expressing GPC3 by flow cytometry.



CD16 and NKp46 expression in PBNK cells and GPC3 expression in Hep3B tumor cells analyzed by flow cytometry.

FIGURE 3: CYT-303 FLEX-NK Engager shows dose-dependent PBNK redirected cytolysis, degranulation, and cytokine production against Hep3B tumor cells

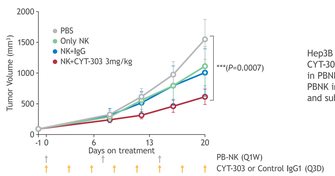


CYT-303 redirected higher PBNK mediated cytolysis of Hep3B tumors compared to mAbs directed against GPC3 or NKp46 at the indicated antibody concentrations at a fixed E/T=1 for 5 hrs as assessed by flow cytometry using a cell viability dye. Dose dependent CYT-303 cytolysis of Hep3B tumors was observed that peaked between 0.4-2 µg/ml concentration.

CYT-303 redirected PBNK cell degranulation in the presence of Hep3B was evaluated using an anti-CD107a antibody by flow cytometry. Dose dependent CYT-303 degranulation peaked around 2 µg/ml concentration. CYT-303 showed higher PBNK degranulation compared to mAbs directed against GPC3 or NKp46 alone suggesting co-engagement of NKp46 and GPC3 leading to an effective immunological synapse is required for optimal NK cell cytotoxicity.

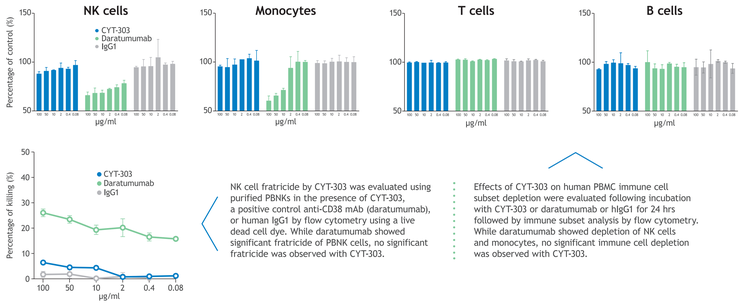
CYT-303 treatment induced higher % of IFN-γ and TNF-α production by PBNK cells compared to GPC3 and NKp46 mAbs alone following incubation with Hep3B cells at E/T=1 for 5 hrs. Cytokine production was assessed by intracellular staining for IFN-γ and TNF-α by flow cytometry.

FIGURE 4: CYT-303 inhibits tumor growth in PBNK injected NSG-IL15 mice bearing Hep3B tumors



Hep3B subcutaneous tumors were established in NSG-IL15 mice. CYT-303 or control IgG1 were administered q3d by intraperitoneal injections in PBNK injected NSG-IL15 mice bearing subcutaneous Hep3B tumors. PBNK injections were administered intratumorally (first injection) and subsequently (second and third injections) by intravenous injections.

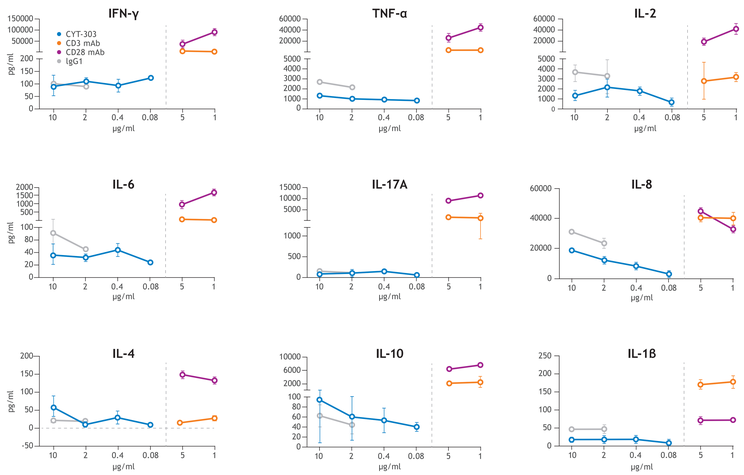
FIGURE 5: CYT-303 has minimal impact on immune subset populations or NK cell fratricide



NK cell fratricide by CYT-303 was evaluated using purified PBNKs in the presence of CYT-303, a positive control anti-CD38 mAb (daratumumab), or human IgG1 by flow cytometry using a live dead cell dye. While daratumumab showed significant fratricide of PBNK cells, no significant fratricide was observed with CYT-303.

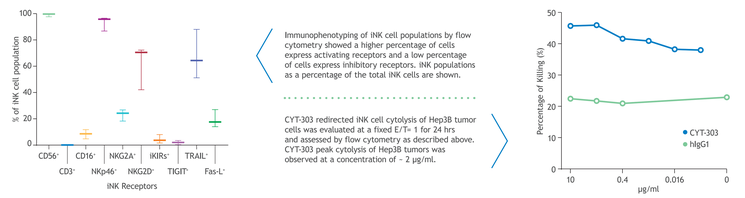
Effects of CYT-303 on human PBMC immune cell subset depletion were evaluated following incubation with CYT-303 or daratumumab or hIgG1 for 24 hrs followed by immune subset analysis by flow cytometry. While daratumumab showed depletion of NK cells and monocytes, no significant immune cell depletion was observed with CYT-303.

FIGURE 6: CYT-303 shows minimal cytokine release in the human PBMC *in vitro* assay



The potential of CYT-303 to induce cytokine release was evaluated in the human PBMC assay following incubation with CYT-303, anti-CD3, CD28 mAbs (TGN1412), or hIgG1 for 48 hrs and supernatants were tested for the presence of cytokines by multiplex ELISA assay. While robust cytokine release was observed with anti-CD3 and anti-CD28 mAbs, no significant cytokine release was observed with CYT-303.

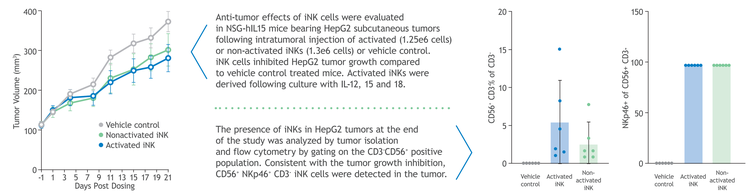
FIGURE 7: iNK cells express multiple activation receptors and show cytotoxic activity against Hep3B tumors



Immunophenotyping of iNK cell populations by flow cytometry showed a higher percentage of cells express activating receptors and a low percentage of cells express inhibitory receptors. iNK populations as a percentage of the total iNK cells are shown.

CYT-303 redirected iNK cell cytolysis of Hep3B tumor cells was evaluated at a fixed E/T= 1 for 24 hrs and assessed by flow cytometry as described above. CYT-303 peak cytolysis of Hep3B tumors was observed at a concentration of ~ 2 µg/ml.

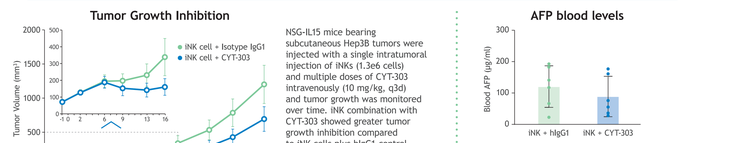
FIGURE 8: Single dose of iNK cells mediated HepG2 tumor growth inhibition in NSG-hIL15 mice



Anti-tumor effects of iNK cells were evaluated in NSG-hIL15 mice bearing HepG2 subcutaneous tumors following intratumoral injection of activated (1.25e6 cells) or non-activated iNKs (1.3e6 cells) or vehicle control. iNK cells inhibited HepG2 tumor growth compared to vehicle control treated mice. Activated iNKs were derived following culture with IL-12, 15 and 18.

The presence of iNKs in HepG2 tumors at the end of the study was analyzed by tumor isolation and flow cytometry by gating on the $CD3^-CD56^+$ positive population. Consistent with the tumor growth inhibition, $CD56^+NKp46^+CD3^-$ iNK cells were detected in the tumor.

FIGURE 9: The combination of iNKs plus CYT-303 showed greater Hep3B tumor growth inhibition compared to iNKs alone in the NSG-IL15 tumor model



NSG-IL15 mice bearing subcutaneous Hep3B tumors were injected with a single intratumoral injection of iNKs (1.3e6 cells) and multiple doses of CYT-303 intravenously (10 mg/kg, q3d) and tumor growth was monitored over time. iNK combination with CYT-303 showed greater tumor growth inhibition compared to iNK cells plus hIgG1 control starting from day 6 post dosing through the end of the study.

Alpha-fetoprotein (AFP) biomarker levels at the end of the study at day 27 in blood were evaluated by ELISA. Consistent with the tumor growth inhibition observed following treatment with iNK cells plus CYT-303, reduced blood AFP levels were detected in mice compared to the mice receiving iNK cells plus hIgG1.

SUMMARY

- CYT-303 is a tetravalent human IgG1 multifunctional NK cell engager antibody with a flexible linker that allows for simultaneous binding to GPC3 and NKp46 on opposing tumor and NK cells respectively.
- CYT-303 shows dose-dependent PBNK and iNK redirected degranulation and cytolysis of Hep3B tumors. Peak cytolysis of Hep3B tumors was observed between 0.4-2 µg/ml.
- CYT-303 treatment in PBNK injected NSG-IL15 mice showed greater Hep3B tumor growth inhibition compared to hIgG1 isotype control.
- iNK cells express multiple activating receptors and few inhibitory receptors compared to PBNKs and consistent with this observation show significantly more potent Hep3B tumor cytolysis.
- Combination of iNK cells and CYT-303 showed greater Hep3B tumor cytolysis compared to iNK cells alone *in vitro*.
- Intratumoral administration of iNK cells to NSG-hIL15 mice bearing subcutaneous HepG2 tumors showed tumor growth inhibition. $CD56^+/NKp46^+$ iNK cells were present in the tumor at end of study.
- iNK cells administered intratumorally in combination with CYT-303 via intravenous injection to NSG-IL15 mice bearing subcutaneous Hep3B tumors showed greater tumor growth inhibition compared to iNK cells plus hIgG1. Concomitant reductions in blood AFP biomarker were observed in these animals.
- CYT-303 *in vitro* safety studies with purified NK cells and human PBMCs showed no significant NK cell fratricide, depletion of immune cells or cytokine release while T cell agonist anti-CD3 and CD28 mAbs (TGN1412) readily induced cytokine release.

CONCLUSIONS

- The FLEX-NK™ multifunctional engager antibody CYT-303 directed against NKp46 and GPC3 demonstrated:
  - potent cytotoxicity against HCC tumor cells accompanied by cytokine production;
  - in vivo inhibition of HCC tumor growth.
- iNK cells expressed a favorable combination of multiple activation and few inhibitory receptors that corresponded to more potent cytolytic activity against HCC targets.
- The combination of the FLEX-NK™ and iNK cells demonstrated greater *in vitro* and *in vivo* anti-tumor activity in HCC models with a favorable cytokine release and immune cell subset safety profile in PBMCs *in vitro*.
- These preclinical proof of concept studies with CYT-303 alone or in combination with iNK cells in HCC warrants clinical development.