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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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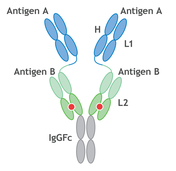
ABSTRACT

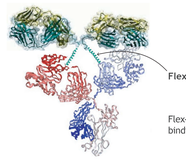
Glypican-3 (GPC3) is highly expressed in multiple solid tumors including HCC while it is hardly expressed in adult normal tissues except placenta. GPC3 promotes Wnt-dependent cell proliferation, and its expression is correlated with poor prognosis in HCC. NK cells exhibit innate anti-tumor activity owing 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 the GPC3 were combined on the novel FLEX-NK™ scaffold to create the NK engager CYT-303. CYT-303 has higher affinity for GPC3 compared to NKp46 to skew binding preferentially to GPC3 expressing tumor cells prior to binding NK cells expressing NKp46 to optimize targeted killing. 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.

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 iPSC, 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 activaton receptors including NKp46 and showed good cytotoxic activity against HCC cell line Hep3B. The iNKs also showed anti-tumor activity in NSG-hIL15 mice bearing HCC subcutaneous tumors as demonstrated by the presence of CD56$^+$CD3$^-$, NKp46$^+$, NKG2D$^+$ iNKs in the tumors at day 21 post-intratumoral injection of the iNKs. 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 IgG1 isotype control. Blood alpha fetoprotein (AFP) levels decreased in the CYT-303 plus iNK combination compared to iNKs alone. 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: Proprietary BsAb Technology Leading to Novel Multifunctional FLEX Format



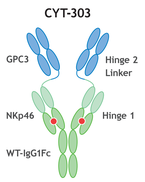
- Tetravalent: higher avidity for target, improved affinity and specificity
- Structure and novel FLEX-linker allow for plug & play design
- Low immunogenicity
- Excellent stability
- FLEX-NK™ construct enhances NK Cell function against target cells
- Manufacturability established



Flex-linker facilitates binding to Antigen AεB

Reference: Golay et al. J. Immunol 2016

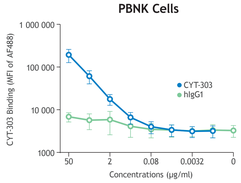
FIGURE 2: CYT-303 Structure and Binding to NKp46 and GPC3 recombinant proteins and PBNK cells and Hep3B tumors expressing NKp46 and GPC3



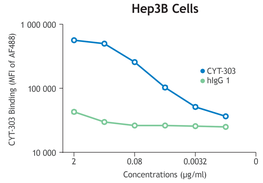
Structure of CYT-303 Flex NK™ tetravalent trifunctional hIgG1 antibody with a flexible linker for simultaneous binding to GPC3 and NKp46.

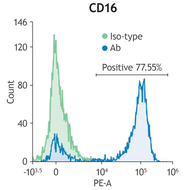
Target	Human Ligands (nM)	Cyno Ligands (nM)	Mouse Ligands (nM)
GPC3	K_D=0.646	K_D=0.273	K_D=0.447
NKp46	K_D=31.7	K_D=3350	K_D= no binding

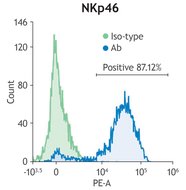
CYT-303 binding affinity analysis to human, cynomolgus monkey and mouse GPC3 and NKp46 by Octet Bio-Layer Interferometry (BLI). CYT-303 binds human GPC3 with a ~50 fold greater affinity than binding to human NKp46 increasing the probability of tumor engagement by NK cells following CYT-303 treatment.



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







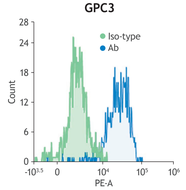
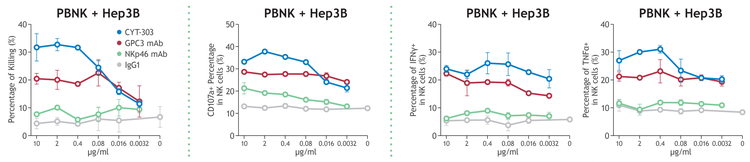


FIGURE 3: CYT-303 FLEX-NK Engager shows dose dependent PBNK redirected cytotoxicity cytolysis, degranulation and cytokine production against Hep3B tumor cells

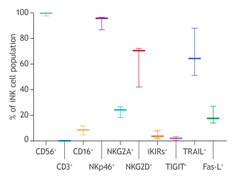


CYT-303 redirected higher PBNK cytolysis of Hep3B tumors compared to single mAbs directed against GPC3 or NKp46 at the indicated CYT-303 concentrations at a fixed E/T=1 for 5 hrs was assessed by flow cytometry using a cell viability dye. Dose dependent CYT-303 cytolysis of Hep3B tumors was observed that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

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

CYT-303 also induced higher % of IFN-γ and TNF-α producing PBNK cells compared to single GPC3 and NKp46 mAbs 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: The iNK cells express multiple activation receptors and show cytotoxic activity against Hep3B tumors



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

CYT-303 redirected iNK cell cytolysis of Hep3B tumors were 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 ~ 2 µg/ml

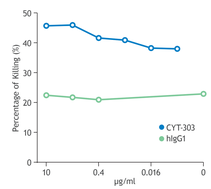
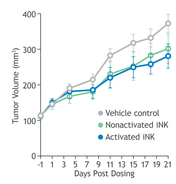


FIGURE 5: iNK cell mediated HepG2 tumor growth inhibition in NSG-hIL15 mice



Anti-tumor effects of iNK cells was evaluated in NSG-hIL15 mice bearing HepG2 subcutaneous tumors following intratumoral injection of activated (1.25e^6 cells) or non-activated iNK's (1.3e^6 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 iNK's in HepG2 tumors at the end of the study was analyzed by tumor isolation and flow cytometry via gating on CD3$^-$CD56$^+$ population. Consistent with the tumor growth inhibition observed, CD56$^+$NKp46$^+$ CD3$^-$ iNK cells were detected.

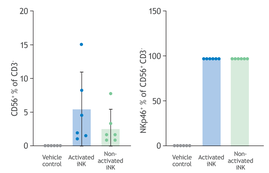
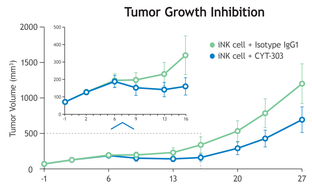
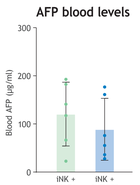


FIGURE 6: The combination of iNKs plus CYT-303 showed greater Hep3B tumor growth inhibition compared to iNKs alone

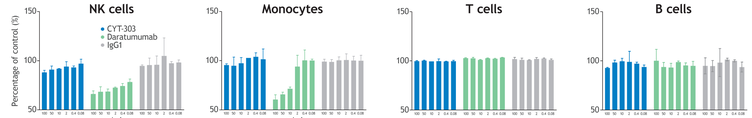


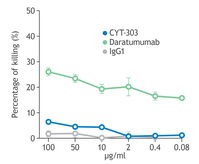
NSG-IL15 mice bearing subcutaneous Hep3B tumors were injected with a single intratumoral injection of iNKs (1.3e6 cells) and multiple doses of CYT-303 intravenously (10 mg/kg, q3d) and tumor growth was monitored over time. iNK combination with CYT-303 showed greater tumor growth inhibition compared to iNK cells plus IgG1 control starting from day 6 post dosing through the end of the study



AFP biomarker blood levels at the end of the study at day 27 were evaluated by ELISA. Consistent with the tumor growth inhibition observed with the iNK combination with CYT-303 reduced blood AFP levels were observed compared to iNK cells alone group of animals

FIGURE 7: Favorable in vitro NK cell fratricide, immune cell and cytokine release profile of CYT-303 with human PBMCs

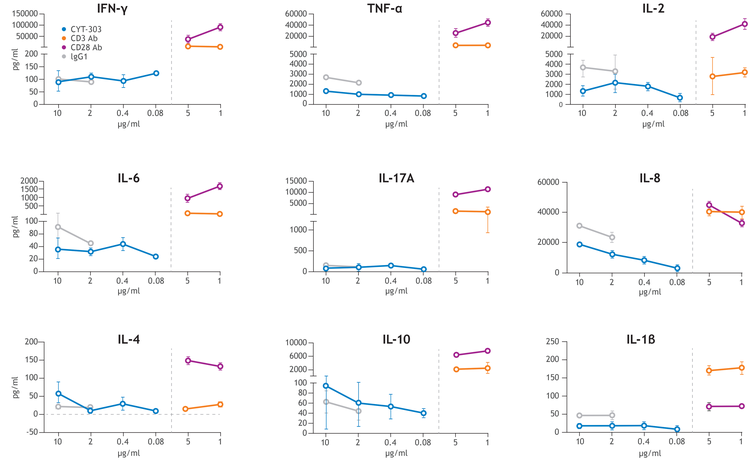




NK cell fratricide by CYT-303 was evaluated using purified PBNK's in the presence of CYT-303 or daratumumab or human IgG by Flow cytometry using the live dead cell dye. While daratumumab showed significant fratricide of PBNK cells, no significant fratricide was observed with CYT-303.

Effects of CYT-303 on human PBMC immune cell subset depletion was 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.

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



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 binds human GPC3 with higher affinity compared to human NKp46 increasing the probability of tumor engagement by NK cells following CYT-303 treatment.
- 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.
- 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.
- 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.
- Combination of iNK cells and CYT-303 showed greater Hep3B tumor cytolysis compared to iNK cells alone in-vitro.
- iNK cells administered intratumorally in combination with CYT-303 via intravenous injection to NSG-IL-15 mice bearing subcutaneous Hep3B tumors showed greater tumor growth inhibition compared to iNK cells alone. Concomitant reductions in blood AFP biomarker were observed in these animals.
- CYT-303 in-vitro safety studies with purified NK cells and human PBMC's 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 in-vitro and in-vivo activity against HCC tumor targets.
- 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 platforms 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-vitro.
- These preclinical proof of concept studies with CYT-303 alone or in combination with iNK cells in HCC warrants clinical development.