Supporting Information

Designing magnetically responsive biohybrids composed of cord blood-derived natural killer cells and iron oxide nanoparticles

Rachel A. Burga†‡, Daud H. Khan§, Nitin Agrawal§, Catherine M. Bollard†‡°, Rohan Fernandes†‡Δ*

† Institute for Biomedical Sciences, George Washington University, Washington, DC 20037, United States
‡ George Washington Cancer Center, George Washington University, Washington, DC 20052, United States
§ Department of Bioengineering, George Mason University, Fairfax, VA 22030, United States
° Center for Cancer and Immunology Research, Children’s National Health System, Washington, DC 20010, United States
Δ Department of Medicine, George Washington University, Washington, DC 20037, United States

*Corresponding Author
rfernandes@gwu.edu
800 22nd St NW
GW Cancer Center – 8th floor
Washington, DC 20052
Phone: 202-994-0899

This supporting information document contains: (1) Supporting Methods and (2) Supporting References

S1
Supporting Methods:

Cell Sources
Umbilical cord blood mononuclear cells were harvested from fresh cord blood units obtained from MD Anderson Cancer Center under approved IRB protocols (Pro00003896) by density gradient separation, and NK cells were isolated by negative selection with the EasySep Human NK Cell Isolation Kit (Stem Cell Technologies, Vancouver, Canada). After 24 hours of activation with 10 ng/mL of human IL-15 (R&D Systems, Minneapolis, MN), NK cells were stimulated with K562 feeder cells, modified to express membrane-bound IL-15 and 41BBL\textsuperscript{1,2} (generously obtained from Baylor College of Medicine (Pro00003869)), which were irradiated at 200 Gy and cultured with NK cells at a 2:1 K562:NK cell ratio. NK cells were cultured in Stem Cell Growth Medium (CellGenix, Germany) supplemented with 200 IU/mL human IL-2, 15 ng/mL human IL-15, 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA), and 1% Glutamax (Gibco, Thermo Fisher Scientific, Waltham, MA) and expanded for 14-21 days. Modified and unmodified K562 cell lines were cultured with IMDM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA), 1% Penicillin-Streptomycin, and 1% Glutamax (Gibco, Thermo Fisher Scientific, Waltham, MA). Neuroblastoma line SHSY5Y was purchased from ATCC (Manassas, VA) and grown in a 1:1 medium of DMEM and F12K medium supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA), and 1% Glutamax (Gibco, Thermo Fisher Scientific, Waltham, MA). We performed HLA (human leukocyte antigen) and STR (short tandem repeat) analysis to ensure cell line accuracy for the SHSY5Y tumor line (Genetica Cell Line Testing, Burlington, NC).\textsuperscript{3}

Nanoparticles and Biohybrid Generation
Streptavidin-conjugated iron oxide nanoparticles were purchased from Nanocs Inc (Boston, MA) and verified for their size (<50 nm) and charge of particles (mV), prior to inclusion in these assays. NK cells were obtained after 14-21 days of expansion, and terminal amines on the NK cell membrane were conjugated to biotin through NHS-ester linkage (Thermo Fisher Scientific, Waltham, MA). NK cell-nanoparticle biohybrids (NK:IONP) were generated by utilizing the high affinity avidin-biotin complex ($K_d = 10^{-15}$ M). Multiple ratios of nanoparticle to NK cell were used: $1\times = 5\times10^{-8}$ mg/cell (50 pg/cell), $0.75\times = 3.75\times10^{-8}$ mg/cell (37.5 pg/cell), $0.5\times = 2.5\times10^{-8}$ mg/cell.
(25 pg/cell), and 0.1× = 5x10⁻⁹ mg/cell (5 pg/cell), with the 1× dose representing an “optimal” surface coating as determined previously.⁴ To determine this optimal surface coating we quantified binding efficiency (bound vs. unbound ratio of IONP) by utilizing the reaction between IONP and Perls Prussian blue. This reaction⁵ results in a blue colloidal suspension that exhibits a characteristic absorption band at 680 nm. By measuring the OD₆₈₀ of the resulting solution from the biohybrid pellet and biohybrid supernatant (obtained after conjugation) and comparing to a standard curve generated with known amounts of IONPs and Perls’ reagent (below), we were able to quantify IONP in our reaction.

![IONP Standard Curve with Perls' Prussian Blue](image)

**Figure S1: Standard Curve of IONP reacting with Perls’ Prussian Blue.** Known amounts (µg) of IONPs were reacted with Perls’ Prussian Blue reagent and the absorbance was read at 680 nm in order to generate a standard curve.

Iron oxide conjugated at the 1× dose resulted in the highest ratio of bound iron oxide to the NK cells vs. iron oxide that remained unattached (unbound) in the supernatant, demonstrating that this dose represents ideal loading capacity. Higher concentrations > 1× dose (e.g. 100 pg/cell) yielded visible aggregation and were therefore not investigated further in this study (data not shown).
Figure S2: Quantification of Iron Oxide in the Biohybrid. Visualization of the blue colloidal suspension resulting from biohybrids (NP pellet; bottom) generated with 0, 5, 25, 37.5, and 50 IONP pg/cell (increasing in concentration from left to right) reacting with Perls Prussian blue reagent.

For flow cytometric assessment of biohybrid persistence over time, we utilized fluorescent avidin to identify the IONP component and fluorescent anti-CD56 antibody to identify NK cell component of the biohybrid. We titered the amount of fluorescent avidin on the IONPs in order to determine the working concentration of avidin that is simultaneously able to achieve robust avidin attachment and prevent fluorescence saturation, and determined labeling IONPs with 100 µg fluorescent avidin/mg IONP was ideal. AlexaFluor488-avidin (Life Technologies) was conjugated to iron oxide nanoparticles at a ratio of 100 µg avidin / 1 mg IONP. We identified this ideal ratio by assessing the mean fluorescence intensity of iron oxide nanoparticles conjugated with increasing amounts of AlexaFluor488-avidin (0, 10, 50, 100, and 300 µg). We found that the avidin fluorescence reached saturation >100 µg, and that conjugating 300 µg of avidin did not provide added benefit.

Figure S3: Avidin Fluorescence. AlexaFluor488-avidin was conjugated to iron oxide nanoparticles at 0, 10, 50, 100, and 300 µg avidin per 1 mg iron oxide nanoparticle, and fluorescence was assessed by flow cytometry.
**Characterization of the Biohybrid**

For scanning electron microscopy, samples of unconjugated NK cells and NK:IONP biohybrids were prepared by fixing on coverslips with 2.5% glutaraldehyde, 1% paraformaldehyde, and 0.12 M sodium cacodylate buffer in distilled water, and sequentially dehydrated with ethanol. Dehydrated samples were dried to their critical point with the Tousimis 931 CPD (Tousimis, Rockville, MD) and platinum sputter coated with the Cressington 208HR Sputter Coater (Cressington Scientific, Watford, UK), prior to imaging on the FEI Teneo LV SEM field emission scanning electron microscope (FEI, Thermo Fisher Scientific, Waltham, MA). For assessment of NK cell phenotype, unconjugated and conjugated NK cells were stained with antibodies specific for NKp30, NKG2D, NKp44, CD16, PD1, CD56, and CD3. Antibodies were conjugated to FITC, PE, PerCP, APC, APC-Cy7, Pe-Cy7, or PerCP-Cy5.5 (BD Biosciences, Franklin Lakes, NJ). Samples were run on the Accuri C6 (BD Biosciences, Franklin Lakes, NJ) or CytoFLEX S (Beckman Coulter, Indianapolis, IN) flow cytometers and analysis conducted using Flow Jo 7.6.5 (FlowJo LLC, Ashland, OR). Voltages were set based on unstained cells, compensation calculated using single-stained controls, positive staining was defined with fluorescence minus one (FMO) controls, and mean fluorescence intensity calculated. To determine the cytolytic properties of unconjugated NK cells and the NK:IONP biohybrid in various conditions, flow-based cytotoxicity assays were performed. Effector cells were incubated with labeled target cells (Cell Trace Far Red, Thermo Fisher Scientific, Waltham, MA) at 40:1, 20:1, 10:1, and 5:1 effector:target ratios for 4 hours in triplicate in round-bottomed 96-well plates. Target cells were incubated in media alone or in 5% Triton X-100 (Sigma, St. Louis MO) to determine spontaneous and maximum release, respectively, and dye dilution was obtained by flow cytometry of fixed samples. The percent killing was determined by the following formula: \(\frac{\text{experimental} - \text{spontaneous dilution}}{\text{maximum} - \text{spontaneous dilution}} \times 100\%\). For transwell cytotoxicity assays, experiments were conducted identically to above, except target cells were placed in the main well, with effector cells (NK or NK:IONP) were added into the transwell insert above.

**Functional Assessment of the Biohybrid**

For transwell assays, 5 µm pore transwell inserts were used, and wells were filled with cell medium prior to addition of NK or NK:IONP into the top of the insert. Individual magnets were adhered at the bottom of each well to generate a vertical magnetic gradient for each transwell condition, and
magnetic fields were applied for 15 minutes. For establishing the external magnetic field, individual 5754 Gauss neodymium block magnets (3/16” x 3/16” x 3/16”, K&J Magnetics, Inc. Pipersville, PA) were utilized. This field strength (0.57 T) is significantly lower than the field strength used for clinical MRI magnets (1.5 - 7 T) that have excellent safety profiles for use in humans. In addition, time scales for applying the magnet (typically 30 minutes or less) are comparable to clinical MRI time scales for diagnostic imaging, further supporting our application with this neodymium magnet. For the experiments described within this study, the magnet was kept < 5 mm from the cells. For generating a 3D tumor gel model, SHSY5Y tumors were cultured in a 2% agarose IV solution, and 3 mm cylindrical sections were isolated with a biopsy punch (Sklar Surgical Instruments, West Chester, PA). Tumor gels were placed in gasket-sealed slides, and NK or NK:IONP suspension was added adjacent to the tumor gel. Individual magnets were placed to the left of tumor gels to create a magnetic gradient directing towards the tumor gel. The 3D tumor gels were digested, and protein was isolated for molecular assessment of perforin and granzyme gene expression by qPCR. Briefly, tumor gels were liquefied and RNA was extracted using the RNA Isolation kit according to manufacturer’s instructions (Zymo Research, Irvine, CA). cDNA was prepared from 1000ng of isolated RNA by performing PCR amplification with RT buffer, dNTP Mix, MultiScribe RT, RNAse inhibitor, random primers, and nuclease free water according to the High Capacity RT cDNA kit (Thermo Fisher Scientific, Waltham, MA). 50ng of cDNA was then combined with Taqman MasterMix and gene expression assays (containing forward primer, reverse primer, and probes) for human perforin (FAM-MGB probe) or granzyme B (FAM-MGB probe), which was multiplexed with GAPDH (VIC-MGB) as a housekeeping gene (all Taqman assays from Thermo Fisher Scientific, Waltham, MA). All samples were analyzed in triplicate, and individual sample results were normalized to GAPDH gene expression in each sample.

**Microfluidic Device Fabrication and Biohybrid assays**

For generating the vascular-mimetic microfluidic device we used previously established soft lithography procedures were used to fabricate the microfluidic devices. Primary designs, adapted from Roberts et al., were prepared using AutoCAD software (AutoDesk Inc, San Rafael, CA) and subsequently translated onto a master wafer with SU-8 photoresist utilizing the lithography system Dilase 250 (Kloe, France). A 50 µm thick SU-8 was spin-coated onto the wafer, followed
by pre-baking, direct write lithography, post-baking and resist-development using standardized procedures. PDMS pre-polymer was prepared (thoroughly mixing the base and curing agent in 10:1 w/w) and poured onto the mold, degassed and thermally cured for 90 minutes at 70°C. The cured PDMS devices were carefully cut using a scalpel and peeled. An 18-gauge flat-end needle was used to make the inlets and outlets. This was followed by sonication in isopropyl alcohol for 30s to remove debris. Once dry, the devices were irradiated with oxygen plasma (channel side up) and irreversibly bonded to a glass slide.

Prior to cell introduction, the cell-loading channels were infused with 0.2% gelatin from porcine skin (Sigma-Aldrich Co., St. Louis, MO) for 30 minutes, then treated with 100 µg/ml fibronectin solution in PBS (Sigma-Aldrich Co., St. Louis, MO) and left overnight. The devices were washed with PBS and the bottom channel was sealed. The unconjugated NK cells or NK:IONP biohybrids were then infused into the top flow channel at constant flow rates (0.1 - 1 µL/min) for specific time intervals respectively using an automated syringe pump (Fusion 100, Chemyx, Stafford, TX), to mimic physiologically relevant environments. Biologically, the microvasculature size and pattern within tumors varies according to both cancer type and location. Mean diameters of blood vessels in glioblastoma were reported to be approximately 50 µm whereas those in malignant nerve sheath tumors can grow larger than 200 µm. In accordance with the variable mean diameter observed in a tumor, shear stress within these systems varies accordingly. The microfluidic device used in this study has been previously characterized by several groups to investigate three-dimensional microvascular networks (µVNs) and 3D cell migration in cancer cells. In this device, the width of the inlet channel was 250 µm (similar to mean diameters of reported blood vessels in tumors). The flow rate of between 0.1-1 µL/minute in turn generated a shear stress of up to 0.25 dyne/cm². These correspond to physiological shear stress values reported by other groups. Alternate reports that utilize microfluidic devices to study cell migration enact similar characteristics (PDMS polymeric devices, fibronectin coating) and size parameters of chambers ~200-400 µm in width. Individual magnets were placed below the bottom chamber to create a magnetic gradient perpendicular to the channel and flow of NK cells in the top chamber. The movement of the both labeled control and modified NK cells was monitored using the EVOS-FL Auto Cell Imaging System (ThermoFisher Scientific, Waltham, MA, USA). An onstage incubator
was used to maintain necessary incubation conditions and the live movements were recorded using custom software.

**Statistics**

All experiments were performed in duplicate or triplicate with 3+ donor NK cell lines, and data is presented with error bars indicating the standard error of the mean. Data was analyzed using GraphPad Prism software (GraphPad, La Jolla, CA) and comparisons between NK and NK:IONP groups were performed using Student’s t-test or Chi-squared tests, with p<0.05 as considered significant and denoted with an asterisk. The biohybrid schematic model was generated using Biorender (Toronto, Canada).

**Supporting References**

1. Cho D, Campana D. Expansion and activation of natural killer cells for cancer immunotherapy. Korean J Lab Med 2009;29:89-96.
2. Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res 2009;69:4010-7.
3. Reid Y, Storts D, Riss T, Minor L. Authentication of Human Cell Lines by STR DNA Profiling Analysis. In: Sittampalam GS, Coussens NP, Brimacombe K, et al., eds. Assay Guidance Manual. Bethesda (MD)2004.
4. Burga RA, Patel S, Bollard CM, CR YC, Fernandes R. Conjugating Prussian blue nanoparticles onto antigen-specific T cells as a combined nanoimmunotherapy. Nanomedicine (Lond) 2016;11:1759-67.
5. Boutry S, Forge D, Burtea C, et al. How to quantify iron in an aqueous or biological matrix: a technical note. Contrast Media Mol Imaging 2009;4:299-304.
6. Xia Y, Whitesides GM. Soft lithography. Angewandte Chemie International Edition 1998;37:550-75.
7. Roberts SA, Waziri AE, Agrawal N. Development of a single-cell migration and extravasation platform through selective surface modification. Analytical chemistry 2016;88:2770-6.
8. Srivastava Y, Loscertales I, Marquez M, Thorsen T. Electrospinning of hollow and core/sheath nanofibers using a microfluidic manifold. Microfluidics and Nanofluidics 2008;4:245-50.
9. Khan DH, Roberts SA, Cressman JR, Agrawal N. Rapid Generation and Detection of Biomimetic Oxygen Concentration Gradients In Vitro. Scientific reports 2017;7:13487.
10. Forster JC, Harriss-Phillips WM, Douglass MJ, Bezak E. A review of the development of tumor vasculature and its effects on the tumor microenvironment. Hypoxia (Auckl) 2017;5:21-32.
11. Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 2004;64:3731-6.
12. Chen MB, Whisler JA, Jeon JS, Kamm RD. Mechanisms of tumor cell extravasation in an in vitro microvascular network platform. Integr Biol (Camb) 2013;5:1262-71.
13. Jeon JS, Bersini S, Gilardi M, et al. Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. Proc Natl Acad Sci U S A 2015;112:214-9.
14. Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. Lab Chip 2013;13:1489-500.
15. Schwarz J, Bierbaum V, Merrin J, et al. A microfluidic device for measuring cell migration towards substrate-bound and soluble chemokine gradients. Sci Rep 2016;6:36440.
16. Li J, Zhu L, Zhang M, Lin F. Microfluidic device for studying cell migration in single or co-existing chemical gradients and electric fields. Biomicrofluidics 2012;6:24121-2412113.
17. Butler KL, Ambravaneswaran V, Agrawal N, et al. Burn injury reduces neutrophil directional migration speed in microfluidic devices. PLoS One 2010;5:e11921.