Mechanisms of action of a killed, bacteria-based, multiple immune receptor agonist (Decoy platform) in development for pulsed anti-tumor immunotherapy

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Background

Activation of immune receptors, such as Toll-like (TLR), NOD-like (NLR) and Stimulator of Interferon Genes (STING) is required for efficient innate and adaptive immunity. Gram-negative bacteria (G-NB) contain multiple TLR, NOD and STING agonists. Potential utility of G-NB for cancer immunotherapy is supported by observations of tumor regression in the setting of infection and Coley's Toxins. Coley reported that intravenous (i.v.) administration was likely most effective but produced toxicity. The discovery of TLRs and their agonists, particularly the potent/multi-functional TLR4 agonist lipopolysaccharide (LPS)-endotoxin, comprising ~75% of the outer membrane of G-NB, suggests that it may be both a critical active ingredient and responsible for dose-limiting toxicity of i.v. G-NB. We have produced killed, intact bacteria products from non-pathogenic G-NB with ~90% reduction of LPS activity. One product, Decoy10, contained TLR2,4,8,9, NOD2 and STING agonist activity and exhibited reduced i.v. toxicity in mice and rabbits relative to unprocessed cells. Decoy10 and closely related Decoy20 produced single agent activity or combination-mediated durable regressions, with immunological memory, in mice with syngeneic or human breast, colorectal, hepatocellular, pancreatic carcinomas or non-Hodgkin's lymphoma. Regressions were observed in combination with chemotherapy, a non-steroidal anti-inflammatory drug, anti-PD-1, or rituximab, were associated with induction of 18-26 plasma cytokines/chemokines, activation of innate and adaptive immune pathways in tumors, and were dependent on NK, $CD4^+$ and $CD8^+$ T cells (1). These and preliminary clinical results demonstrating rapid clearance of Decoy20 with transient induction of >50 plasma cytokines/chemokines (2) are supportive of a "pulse-prime" mechanism, whereby Decoy bacteria produce transient, but broad innate and adaptive immune activation.

Results Summary

We have analyzed immune activation by Decoy bacteria *in vitro* using human peripheral blood mononuclear cells (PBMCs). Decoy10 induced monocyte-derived dendritic cell maturation, Th1 $CD4^+$ T cell polarization and inhibited Treg production. Decoy10 also induced activation markers in Natural Killer (NK), Natural Killer T (NKT), NKT-Like, $CD4^+$ T, and $CD8^+$ T cells, and induced production of activated $CD4^+CD8^+$ double positive T cells. Some of the results were dependent on or enhanced by Decoy10 combination with IL-2. Finally, Decoy10 enhanced PBMC-mediated tumor cell killing. The results demonstrate that Decoy bacteria stimulate indirect or direct maturation, polarization or activation of cellular mediators of innate and adaptive anti-tumor immune responses, inhibit an immune-suppressive mechanism, and enhance immune cell killing of tumor cells.

Methods

Decoy10 was prepared as described (1). Experiments were carried out at HD Biosciences (San Diego, CA) in triplicate or duplicate when PBMCs from three donors were tested (96-well format). Frozen human PBMCs were obtained from StemExpress (Placerville, CA) or AllCells (Alameda, CA), or isolated from whole human blood purchased from the San Diego Blood Bank using LymphoPrep/SepMate from StemCell Technologies. Flow cytometry studies were carried out with an Attune Flow Cytometer from ThermoFisher. PBMCs in Complete RPMI medium were used at 2.5x10^5 per well for CD4 polarization, and NK, NKT, NKT-Like and CD4/CD8 T cell activation experiments, and at 1x10^4 per well in tumor cell killing experiments. DC maturation experiments utilized 1.25x10^5 CD14+ monocytes per well derived from human PBMCs with the EasySep™ Human Monocyte Isolation Kit from STEMCELL.

Immature CD209+ DCs were induced by incubating CD14+ monocytes for two days with 500 ng/mL GM-CSF + IL-4. After centrifugation, the cells were then incubated for one additional day in fresh medium containing GM-CSF + IL-4 and maturation cytokines 2,000 ng/mL PGE2, 10 ng/mL IL-6, IL-1β, TNF-α (positive control), or GM-CSF + IL-4 and Decoy10 (10^4 to 10^7 cells per mL). DC maturation was assessed by flow cytometry measurement of surface activation markers CD80, CD83 and CD86.

CD4 T cell polarization analysis was carried out after incubating spun/adhered PBMCs for 6-7 days. Th1 polarization was assessed after incubation without or with 1 µg/mL anti-CD28, 1 µg/mL IL-4, 20 U/mL IL-2 and 20 ng/mL IL-12p70 (positive control), or with 10^5 to 10^8 Decoy10 per mL (± 20 U/mL IL-2 + 1 µg/mL anti-CD28). Th1 polarization was determined via CD4+IFN-γ+ expression. Th2 (positive control with anti-CD28, anti-IFN-γ, IL-2, and IL-4) and Th17 (positive control anti-CD28, anti-IL4, anti-IFN-γ, IL-2, IL-1β, IL-6, IL-21, IL-23, and TGF-β) polarization was also assessed with Decoy10 (data not shown). CD4 Treg polarization was induced by 1 µg/mL anti-CD28, 100 U/mL IL-2 and 5 ng/mL TGF-β, and was determined via CD4+CD25+FoxP3 expression.

Activation of NK (CD56+, CD4-, CD8-), NKT (CD56+, CD4+, CD8-), NKT-Like (CD56+, CD4-, CD8+), $CD4^+$ T (CD56-, CD4+, CD8-), $CD8^+$ T (CD56-, CD4-, CD8+) and CD4+/CD8+ double positive T (CD56-, CD4+, CD8+) cells was induced by incubating PBMCs for two days with 100 U/mL IL-2 (positive control for NK cells), or with 2.5 µg/mL anti-CD28 in wells pre-coated overnight with 100 µL of 5 µg/mL OKT3 anti-CD3 antibody (positive control for all T cell sub-types) + 1x10^7 Decoy10/mL, or with Decoy10 alone. Cell activation was assessed by flow cytometer measurement of surface CD69, CD226, and intracellular IFN-γ, Perforin, Granzyme B using standard methods. Total cell number is also reported for double-positive CD4+CD8+ T cells. Immune activation studies were carried out with three independent PBMC donors (Red, Green, Blue) in duplicate. Results for a donor were not included where there were significant discrepancies between duplicates.

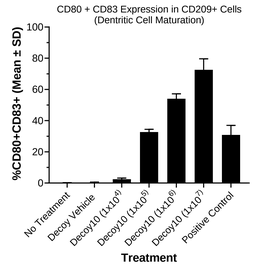
Killing of human MDA-MB-231 breast carcinoma cells (ATCC) by Decoy10 + PBMCs was assessed with IncuCyte™ NucLight™ Red Lentivirus-transfected tumor cells (reagents from Essen Biosciences, Ann Arbor, Michigan). Tumor cells were plated at 2x10^4 and incubated overnight in complete RPMI medium. PBMCs (1x10^4 per well) and/or Decoy10 (3x10^6 per mL) were added, and the incubation was continued for 7 days followed by image analysis using the IncuCyte™ system (Sartorius).

Statistical analysis was carried out using GraphPad Prism software. Triplicate experiments were analyzed using standard, unpaired t-test, and three donor duplicate experiments were analyzed using an unpaired, non-parametric, Mann-Whitney test. All results with Decoy10 alone, or instances where Decoy was not effective by itself, but was effective in combination, produced p values between <0.0001 and 0.035 relative to Decoy Vehicle or each of the individual combination components.

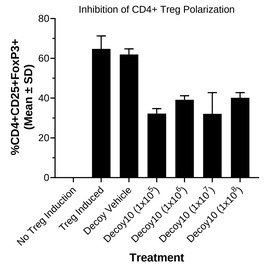
Decoy10 Induced DC Maturation, $CD4^+$ Th1 Polarization, Treg Inhibition, and Activation Markers in NK, NKT, $CD4^+$ and $CD8^+$ T Cells and Enhanced PBMC-Mediated Killing of Tumor Cells

Decoy10 Induces Dendritic Cell Maturation

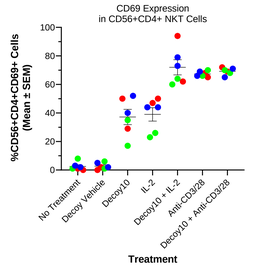
~80-90% of Decoy10-induced double-positive cells were also CD80+CD83+CD86+ (not shown)



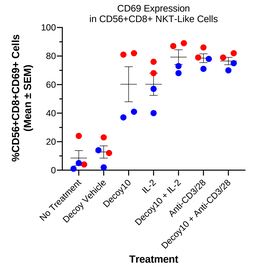
Decoy10 Inhibits Treg Polarization



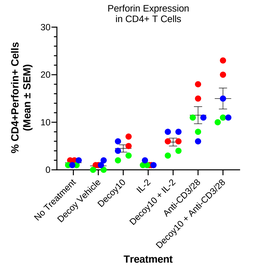
Decoy10 or Decoy10 + IL-2 Induce Activation Markers in NKT cells



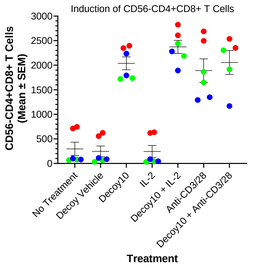
Decoy10 or Decoy10 + IL-2 Induce Activation Markers in NKT-Like cells



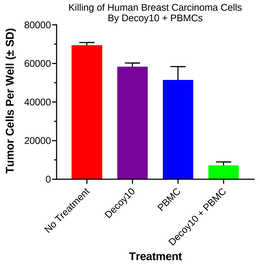
Decoy10 or Decoy10 + IL-2 Induce Activation Markers in CD4 T Cells



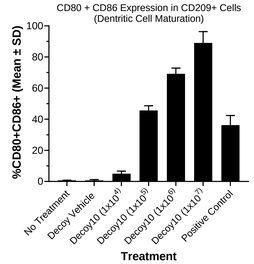
Decoy10 Induces CD4+CD8+ Double Positive T Cells

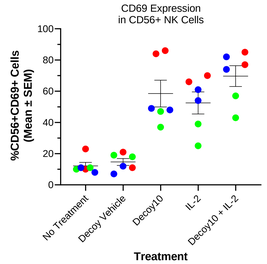


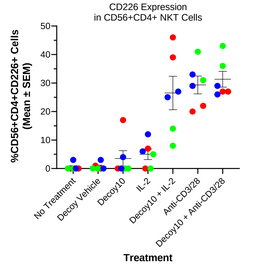
Decoy10 Enhances Killing of Human Breast Carcinoma Cells by PBMCs

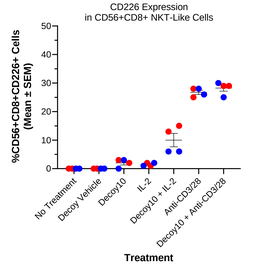


Decoy10 Induces Activation Markers in NK Cells (3 PBMC donors [red, green, blue])

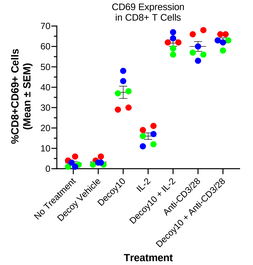




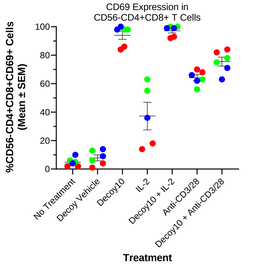




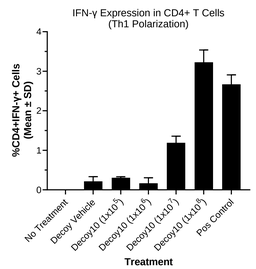
Decoy10 or Decoy10 + IL-2 Induce Activation Markers in CD8 T Cells

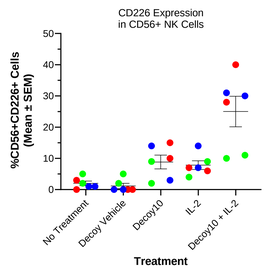


Decoy10 also Induces Activation markers in CD4+CD8+ Double Positive T Cells

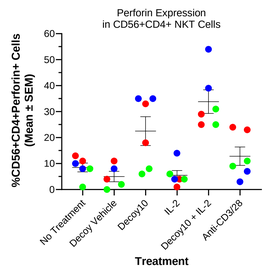


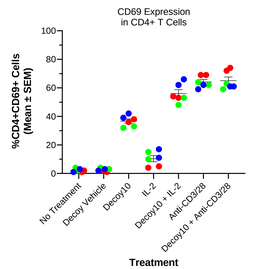
Decoy10 Induces CD4 Th1 Polarization

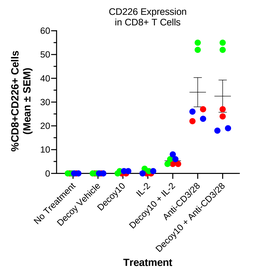


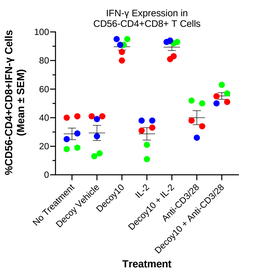


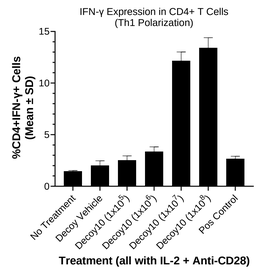
Decoy10 or Decoy10 + IL-2 Induce Activation Markers in CD4 T Cells

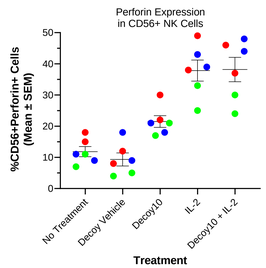


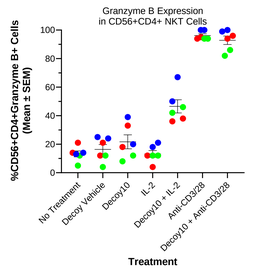


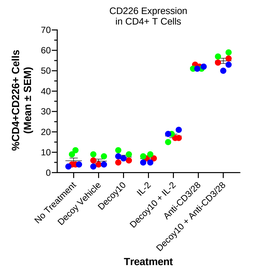


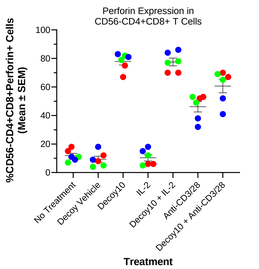












Discussion

Indirect or direct induction of maturation, polarization or activation of multiple innate and adaptive immune cell types, and enhancement of PBMC-mediated tumor cell killing by Decoy10 is highly consistent with our pre-clinical *in vivo* data demonstrating that tumor eradication by Decoy10 combination therapy is dependent on NK, CD4 T and CD8 T cells, and is associated with innate and adaptive immune pathway activation (1).

The new data are also highly consistent with our preliminary Phase 1 clinical trial results, where we have observed broad plasma cytokine and chemokine-associated immune activation after a single dose of Decoy20 (2).

The current data also suggest that Decoy bacteria may produce additive or synergistic effects with IL-2 in some settings.

References

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2. Michael J Newman, Jeffery A Nieves, Gary B Gordon, Diana L Hanna, Angela T Alistar, Mohammed N Al Hallak, Roger J Waltzman. Preliminary results of an in progress, first-in-human Phase 1 study of Decoy20, an intravenous, killed, multiple immune receptor agonist bacterial product in patients with advanced solid tumors. J. Immunother. Cancer 2023; 11 (Suppl2) 782-E: A1770.

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