A systemically administered killed bacteria-based multiple immune receptor agonist for pulsed anti-tumor immunotherapy (induction of tumor eradications, innate and adaptive pathways and immunological memory in multiple pre-clinical models)

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Background / Methods / Results

BACKGROUND) Systemic activation of multiple immune receptors, such as Toll-like receptors (TLR), NOD-like receptors (NLR) and Stimulator of Interferon Genes (STING) is essential for efficient innate and adaptive immune responses. Attempts to use single receptor agonists for advanced cancers have not produced approved products. Limitations include insufficient immune activation and dose-limiting toxicities associated with continuous systemic exposure. Gram-negative bacteria (G-NB) contain multiple TLR, NOD and STING agonists. The potential utility of G-NB for cancer immunotherapy is supported by the long-standing observation of tumor regression in the setting of infection and Coley's Toxins. Coley reported that intravenous (i.v.) administration of his killed bacterial preparation was likely most effective but produced significant toxicity. The discovery of TLRs, particularly the broad/potent innate and adaptive immune-stimulating activities of the TLR4 agonist lipopolysaccharide (LPS)-endotoxin suggest that it may be both a critical active ingredient and a dose-limiting component of i.v. G-NB. Therefore, LPS-endotoxin activity was selectively reduced by ~90% to produce a 100% killed, non-pathogenic, multi-immune agonist G-NB product for systemic cancer immunotherapy.

METHODS) Product manufacturing (Decoy10) was carried out at Molecular Diagnostic Services (San Diego, CA). Non-pathogenic, diaminopimelic acid (DAP) auxotrophic *E. coli* strain 2617-143-312 (Migula) Castellani and Chalmers (ATCC 13070) was grown in LB/Miller broth supplemented with 0.5% glucose, 1 mM DAP and 2 mM MgCl2. Late log phase cells were harvested, washed twice with LB/Miller broth, 0.1 mM DAP, 20 mM MgCl2 at 4°C by centrifugation and resuspended at 1x10^10 cells per ml. LPS activity was reduced by treating cells with 1 mg/ml polymyxin B (PMB) for 1 hour at 4°C, with gentle stirring. Cells were washed three times with 4°C PBS, pH 7.5, 20 mM MgCl2, resuspended at 1x10^10 per ml, and killed by incubation with 1% glutaraldehyde (GA) for 1 hour with gentle stirring at 4°C. Cells were washed three times in above incubation medium without GA, resuspended in 50% PBS, pH 7.5, 2 mM MgCl2, 12% trehalose (freezing medium), flash-frozen and stored at -80°C. Viability and strain confirmation were assessed by plating efficiency ±DAP. LPS-endotoxin activity was determined by Limulus Amebocyte Lysate (LAL) assay. Cellular integrity was assessed by electron microscopy (EM) and/or optical microscopy after Gram staining. Pyrogenicity was assessed in a standard rabbit rectal temperature test at Pacific BioLabs, Inc. (Hercules, CA). TLR, NOD and STING agonist activity was assessed using HEK293 reporter gene assays at InvivoGen (San Diego, CA). Induction of cytokine and chemokine secretion by human peripheral blood mononuclear cells (hPBMCs) was carried out at Eurofins Panlabs (St. Charles, MO) using Luminex technology. *In vivo* anti-tumor assays were carried out by AntiCancer, Inc., (San Diego, CA) and Crown Bioscience (Beijing, China and San Diego, CA) with ~7-week-old female BALB/c, C57BL/6 or C.B17/SCID mice. Decoy10 was washed by centrifugation and resuspended in PBS, 2 mM MgCl2 prior to *in vivo* i.v. administration. Anti-tumor activity and mechanism of action was assessed in orthotopic murine CT26 colorectal carcinoma (tumor fragments sewn onto secum), subcutaneous (s.c.) murine EMT6 breast carcinoma transfected with human HER2 receptor, s.c. murine H22 hepatocellular carcinoma (HCC), s.c. A20 murine non-Hodgkin's lymphoma (NHL) and s.c. human Ramos NHL. NanoString gene expression analysis was carried out at WuXi AppTec (Shanghai, China). Mouse plasma cytokine analysis was carried out at Crown Bioscience (Beijing, China) using Luminex technology.

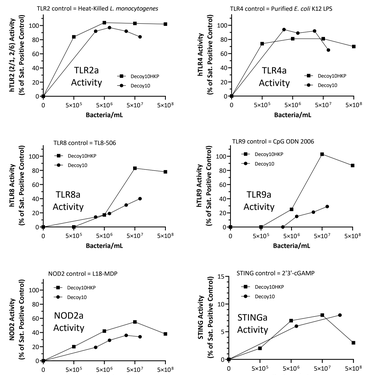
RESULTS) Decoy10 exhibited reduced LPS-endotoxin activity and i.v. toxicity (pyrogenicity) relative to unprocessed cells and was shown to contain agonists for TLR2/1, TLR2/6, TLR4, TLR8, TLR9, NOD2 and STING. Surprisingly, despite significant reduction in LPS-endotoxin activity and i.v. toxicity, Decoy10 induced secretion of similar or higher levels of most cytokines by hPBMCs, compared to unprocessed bacteria. Higher cytokine induction was also observed compared to monospecific TLR agonists. Administration of 2 to 7 weekly or twice weekly i.v. doses of Decoy10 to mice with established s.c., orthotopic or metastatic syngeneic breast, colorectal, hepatocellular, pancreatic carcinomas, s.c. syngeneic NHL or human NHL xenografts was well-tolerated and produced single agent anti-tumor activity, single agent durable tumor regressions and/or combination-mediated durable tumor regressions, with induction of innate and adaptive immunological memory. Regressions were observed, without significantly increased toxicity, when Decoy10 was combined with low-dose chemotherapy (LDC), a non-steroidal anti-inflammatory drug (NSAID), anti-PD-1 therapy or rituximab. Tumor regressions were associated with activation of innate and adaptive immune pathways in tumors after one dose of Decoy10 and were mediated by NK, CD4+ and CD8+ T cells.

Results

Table 1. Decoy treatment kills *E. coli* and significantly reduces LPS-endotoxin activity and *in vivo* pyrogenicity

Treatment	Live Bacteria	LPS Endotoxin Activity (LAL Assay)	Pyrogenicity Threshold (Rabbit Assay)
No Treatment	100%	44.7 Units / 10^6 Bacteria	$3x10^4$ Bacteria
Decoy	0%	3.6 Units / 10^6 Bacteria (92% reduction)	$9x10^5$ Bacteria (97% reduction)

Figure 1. Decoy10 bacteria and Decoy10 heat-killed parent bacteria (Decoy10HKP) contain TLR1,2,4,6,8,9, NOD & STING agonist activity



Human embryonic kidney (HEK293) cells transfected with individual immune receptors and containing reporter genes were challenged with Decoy10 or Decoy10HKP bacteria (in triplicate). Results are plotted as a percentage of the saturating positive control TLR agonist (TLRa) activity for each HEK293 cell line.

Results

Table 2. Despite reduced LPS and pyrogenicity, Decoy10 induces hPBMCs to secrete similar or higher levels of most anti-tumor cytokines than untreated bacteria

Secretion by Human PBMCs *In Vitro*	Untreated Bacteria	Decoy-Treated Bacteria (Decoy10)
Anti-Tumor Cytokine	48-hour pg/mL peak (mean of triplicates) at same bacteria dose for each cytokine	
GM-CSF	1,094	1,197
IFNγ	175,866	47,488*
IL-12p70	176	528
IL-23	0	119
TNFα	49,782	77,919

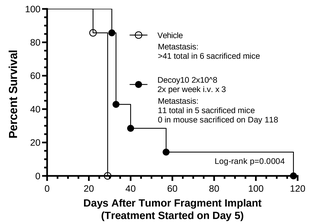
*Peak induction of IFNγ by Decoy10 at higher dose-levels was significantly higher

Table 3. Decoy10 induces hPBMCs to secrete higher levels of anti-tumor cytokines than mono-specific TLR agonists

	CpG ODN (TLR9a)	Poly(I:C) (TLR3a)	R848 (TLR 7/8a)	LPS (TLR4a)	Decoy10 (TLR2,4,8,9a)
Anti-Tumor Cytokine	pg/mL (48-hour full titration peak mean)				
GM-CSF	0	0	87	175	1,197
IFNγ	7	103	31,324	29,416	91,475
IL-12p70	4	18	253	109	528
TNFα	51	208	33,393	24,944	77,919

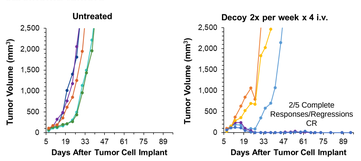
Figure 2. Single agent Decoy10 inhibits metastasis and extends survival of mice with orthotopic CT26 colorectal carcinoma

Tumor fragments were sewn onto the cecum wall of BALB/c mice on day 0 and treatment was started on day 5 with 7 mice per group (tumor cells express green fluorescent protein for metastasis imaging)



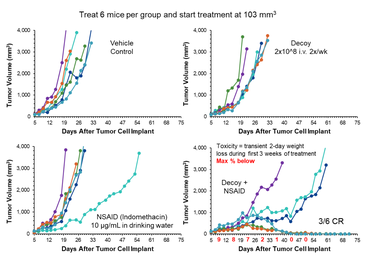
Toxicity – single agent Decoy10 typically produced 5-10% transient weight loss for 1-2 days post-dosing at 2-4x10^8 Decoy10 bacteria per mouse. There was less weight loss with subsequent treatments due to LPS tolerance. The acute (single dose) MTD (no deaths) was 1x10^10 Decoy10 per mouse in separate studies.

Figure 3. Single agent Decoy10 induces regression of s.c. foreign antigen-expressing EMT6-HER2 murine breast carcinoma tumors



EMT6 murine breast carcinoma cells expressing human HER2 receptor were implanted s.c. in BALB/c mice (5 mice per group) and treatment was started when tumors were ~170 mm³. Anti-PD-1 checkpoint therapy produced a similar result (3/5 CR, not shown).

Figure 4. Twice per week i.v. Decoy10 synergizes with daily low-dose oral indomethacin (NSAID) to regress 3/6 established s.c. mouse H22 hepatocellular carcinomas



Results

Figure 5. Combination of 1x per week i.v. Decoy10 with NSAID + anti-PD-1 checkpoint therapy produces 100% tumor regressions with mouse H22 hepatocellular carcinoma tumors

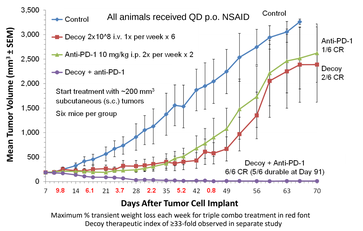


Figure 6. Mice cured by Decoy10 + NSAID + anti-PD-1 and re-challenged with fresh H22 HCC tumor cells reject the tumors demonstrating 100% immunological memory

Nine cured mice from a repeat experiment were re-challenged with HCC tumor cells on Day 91 on the opposite flank from the first challenge

Six naïve mice were challenged with the same tumor cells as the cured mice on the same day

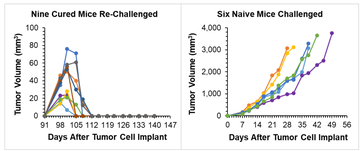


Table 4. Decoy10, NSAID and anti-PD-1 synergistically induce plasma cytokine/chemokine expression in tumor-bearing mice without a significant increase in toxicity

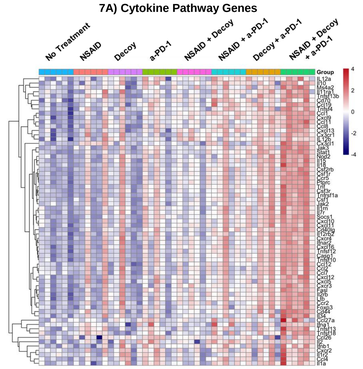
	NSAID	Decoy10	Anti-PD-1	Decoy10 + Anti-PD-1	NSAID + Decoy10	NSAID + Anti-PD-1	NSAID + Decoy10 + Anti-PD-1
Number of HCC Tumor Regressions Per Group in Separate Experiments (Decoy10 1x per week)							
	0 / 6	0 / 6	0 / 6	2 / 6	2 / 6	1 to 2 / 6	4 to 6 / 6
Plasma prepared from mice treated as above 6 and 24 hours after single agent or after second/third agent in combo							
Cytokine / Chemokine	Statistically Significant Cytokine / Chemokine induction Relative to No Treatment (at 6 and/or 24 hours)*						
Eotaxin				6			6
G-CSF				6 / 24	6	6	6
GM-CSF				6 / 24	6	6	6
IFN-gamma				6			
IL-1alpha			6	6 / 24	6	6	6
IL-1beta				6	6	6	6
IL-2				6 / 24	24	24	24
IL-3				6 / 24	24	6 / 24	6 / 24
IL-4				6 / 24	6 / 24	6 / 24	6 / 24
IL-5					6		6 / 24
IL-6				6 / 24	6		6
IL-7							
IL-9			6	6 / 24	6 / 24	6 / 24	6 / 24
IL-10			6	6 / 24			6
IL-12p40				6 / 24	6	6 / 24	6 / 24
IL-12p70				6 / 24	6	6	6
IL-13				6 / 24	6 / 24	6 / 24	6 / 24
IL-15				6 / 24			6 / 24
IL-17			6	6			
LIF					6		
MIP-1b				6	6	6	6
KC	6			6 / 24			6
MCP-1			6	6 / 24	24		6
M-CSF							6
MIP-1alpha	6			6		6	6
MIP-1beta			6	6 / 24			6
MIP-2			6	6 / 24	6 / 24	6 / 24	6 / 24
MIG			6	6 / 24	6 / 24	6	6
Rantes			6	6 / 24	6	6	6
TNF-alpha			6	6 / 24	6	6	6
VEGF							

*At either 6 and/or 24 hours after first treatment / Determined by unpaired, non-parametric, Mann-Whitney t-test (p value < 0.05) / 5 mice per group in all groups

Figure 7. Tumor eradication mediated by Decoy10, NSAID and Anti-PD-1 is associated with induction of cytokine, chemokine, innate and adaptive immune pathways in HCC tumors after one week of treatment (1 dose of Decoy10)

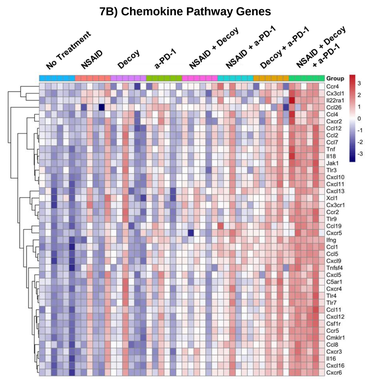
Mice with 200 mm³ established tumors (6 per group) were treated for one week (1 dose of i.v. Decoy10, 2 doses of i.p. anti-PD-1 and/or QD p.o. NSAID). Tumors were isolated, RNA was prepared and subjected to 770 gene NanoString gene expression analysis.

7A) Cytokine Pathway Genes

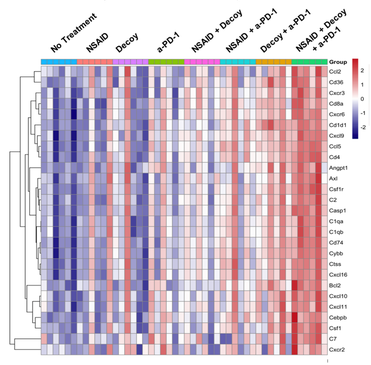


Results

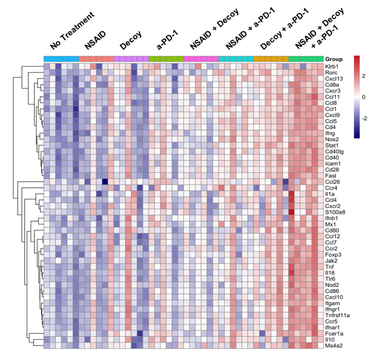
7B) Chemokine Pathway Genes



7C) Innate Immune Pathway Genes



7D) Adaptive Immune Pathway Genes



Combination therapy-mediated tumor eradication was also associated with an increase in tumor inflammation signature (i.e. cold to hot tumor)

A related Indaptus product (Decoy20) is currently being evaluated in a Phase 1 advanced solid tumor trial in the US (NCT05651022)

Acknowledgements: I thank the contract research organizations that carried out the studies, Dr. John Chicca of Molecular Diagnostic Services and Walt Linscott, Jeffrey Meckler and Dr. Deepak Singh of Indaptus Therapeutics for helpful discussions

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Figure 8. Combination of Decoy10 with NSAID and/or anti-PD-1 enhances survival in a mouse model of metastatic pancreatic carcinoma

Pan02 mouse pancreatic carcinoma cells were implanted surgically in the spleens of C57BL/6 mice. Untreated mice developed large tumors in the spleen, liver and pancreas. Single agent i.v. Decoy10 and i.p. anti-PD-1 each produced slight but statistically significant survival enhancements. Combinations with oral indomethacin (NSAID) further extended survival, and the triple combination produced 2 longer-term survivors to at least Day 115.

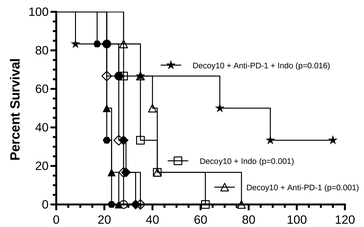
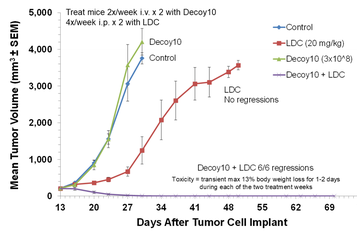


Figure 9. Decoy10 synergizes with low-dose cyclophosphamide (LDC) to eradicate 200 mm³ established s.c. mouse A20 non-Hodgkin's lymphoma (NHL) tumors



Decoy10 + LDC was active & tolerated from 1x10^8 to 1x10^9 Decoy10 per mouse (TI = ~10)

Figure 10. Synergistic eradication of established NHL by Decoy10 + LDC is reproducible, durable and induces 100% immunological memory

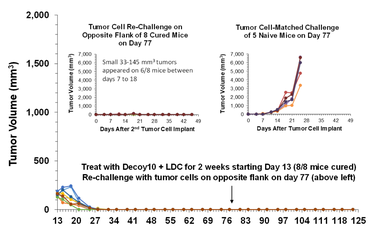


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Figure 11. Depletion of NK or CD4+ T or CD8+ T cells before Decoy10 + LDC treatment prevents high percentage tumor eradication

Indicated groups (6 mice/group) were pre-depleted of NK, CD4+ T and/or CD8+ T cells with commercial antibodies prior to treatment of all groups (average 212 mm³ tumors) with i.v. Decoy10 + i.p. LDC for 2 weeks as in Fig. 9 (immune cell depletion prior to Decoy10 + LDC treatment was confirmed in duplicate groups)

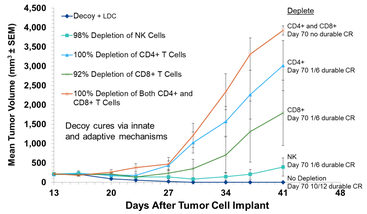


Figure 12. Decoy10 + LDC synergize with rituximab to induce regression of s.c. human Ramos NHL in SCID mice with only an innate immune system (treatment started with average 173 mm³ tumors)

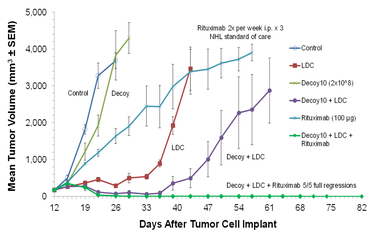
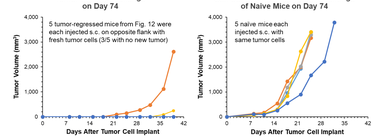


Figure 13. SCID mice with regressed human NHL tumors from Figure 12 (Day 74) reject 3/5 tumor rechallenges demonstrating innate immunological memory



Summary

We have invented novel, multi-TLR, NOD and STING agonist bacteria-based cancer immunotherapies with the following key design characteristics and features:

1) Enhanced i.v. safety due to 90% selective reduction of LPS-endotoxin activity and use of 100% killed, non-pathogenic bacteria

2) Ability to activate both innate and adaptive immune pathways, due to presence of agonists for TLR2 (2/1 and 2/6), TLR4, TLR8, TLR9, NOD2 and STING

3) Ability to activate systemic immune pathways due to predicted passive targeting to liver and spleen

4) Enhanced i.v. safety due to expected rapid clearance (~1 hour), based on published data for bacteria introduced systemically to mice, rabbits and humans (pulsed approach)

Decoy10 inhibited tumor growth, metastasis and induced tumor regressions as a single agent. Regressions were also seen with Decoy10 in combination with an NSAID, anti-PD-1 checkpoint therapy, low-dose chemotherapy (LDC) or LDC plus a targeted antibody. *In vivo* anti-tumor activity was seen with colorectal, breast, hepatocellular, pancreatic carcinomas and non-Hodgkin's lymphomas (murine & human) in pre-clinical models.

Tumor eradication by Decoy10-mediated combination therapy was associated with induction of innate and adaptive immune pathways in tumors after one i.v. dose of Decoy10, involved NK, $CD4^+$ and $CD8^+$ T cells and induced innate & adaptive immunological memory.

Decoy10 was also active in pre-clinical *in vivo* models of chronic HBV and HIV infection (not shown).