Systemic Administration of STING Agonist Antibody-Drug Conjugates Elicit Potent Anti-Tumor Immune Responses with Minimal Induction of Circulating Cytokines

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Poster # 6706

Abstract

STING pathway agonism has emerged as a potential therapeutic mechanism to stimulate an innate anti-tumor immune response. While in principle systemic administration of a STING agonist would have many therapeutic benefits, including the delivery of STING to all tumor lesions, such an approach may be limited by toxicity. Antibody-drug conjugates (ADCs) constitute a proven therapeutic modality that is ideally suited to enable systemic administration without associated toxicity concerns via a targeted delivery strategy.

Herein, we demonstrate that systemically administered STING agonist ADCs have greater anti-tumor activity as well as greatly improved tolerability compared to an intravenously (IV) administered, unconjugated (free) agonist. We generated novel STING agonist ADCs by leveraging our Immunosynthen platform, in which the chemical scaffold for bioconjugation is optimized for the STING agonist, resulting in an ADC that has desirable physicochemical and drug-like properties. We have studied the *in vitro* activity and mechanism of action of STING agonist ADCs in monoculture and co-culture systems. STING agonist ADCs were at least 100-fold more potent in inducing interferon and cytokines as well as tumor cell-killing relative to free agonist.

STING agonist ADCs against several targets (antigens) have been evaluated for anti-tumor activity and pharmacodynamic and pharmacokinetic properties in multiple xenograft and syngeneic models. A single administration of STING agonist ADC resulted in target-dependent, durable, and complete regressions. Importantly, the STING agonist ADC led to an increase in tumor-localized inflammatory cytokines and significant immune cell infiltration, while levels of systemic cytokines remained low. In contrast, IV administered free agonist induced up to 100-fold higher levels of systemic cytokines with concomitant body weight loss but only modest tumor growth delay.

In summary, Immunosynthen represents a novel STING agonist ADC platform. We have demonstrated target-dependent anti-tumor immune responses *in vitro* and *in vivo* for multiple targets, tumor models, and mouse strains. In each case the STING agonist ADC was more active and better tolerated than the IV administered free agonist.

STING Pathway

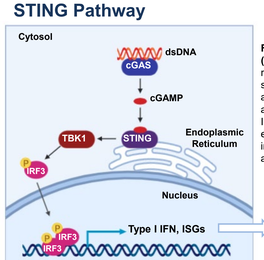


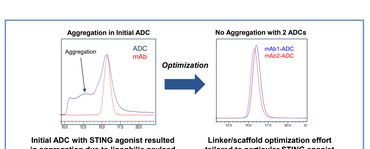
Figure 1. Depiction of the STING pathway (adapted from Corrales et al[1]). cGAS recognizes and binds to cytosolic dsDNA and synthesizes cGAMP, a natural STING agonist that activates STING, which in return activates TBK1-mediated phosphorylation of IRF3 transcription factor, leading to expression of type I interferon and other interferon stimulated genes and eventually to an anti-tumor immune response.

Anti-Tumor Immune Responses

Strategy for Optimal ADC Synthesis

Building ADCs with a modular approach:

- Flexibility in design enables optimization of ADC for optimal pharmacological and pharmacokinetic properties.
- Modular components enable fine-tuning of drug-to-antibody ratio (DAR).
- Amenable to many bioconjugation methods.
- Ultimately the ADC is optimized for the target, antibody, and payload.



Targeted STING Agonist ADC exhibits More Than 100-fold Increased Potency Compared to Free Payload, *In vitro*

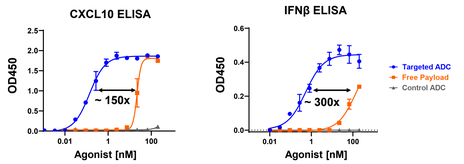


Figure 2. Potent activation of STING pathway by targeted ADC, *in vitro*. Targeted ADC exhibits ~150 – 300x higher potency compared to free payload. Control, non-targeted ADC (non-binding mAb conjugated to the STING agonist) has no significant activity. Assay is based on a co-culture of cancer cells expressing ADC target and immune cells. ADC concentrations are based on payload.

Antigen and FcγR Binding are Essential for Tumor Targeting and Immune Cell Activation by STING Agonist ADCs

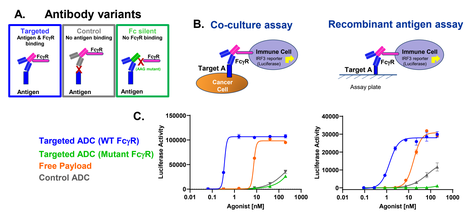
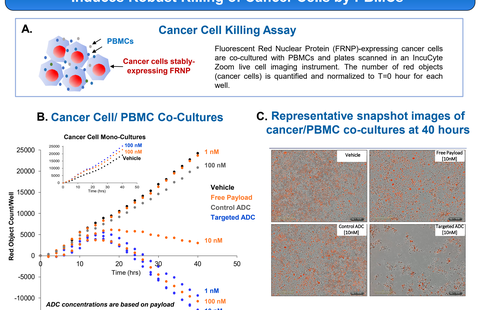


Figure 3. Antigen and FcγR binding are both required for STING agonist ADC activity. A) Antibody variants used to confirm mechanism of immune cell activation by STING agonist ADC. **B)** Depiction of two assays used to confirm requirement for antigen and FcγR for mechanism of immune cell activation by STING agonist ADC, in the presence or absence of cancer cells. **C)** Binding of STING agonist ADC to tumor cell antigen expressed on cell surface or plate bound is required for ADC targeting and STING activation in immune cells. Non-targeted control ADC shows minimal activity at high ADC concentrations only. Mutation introduced in the FcγR binding region of the antibody abrogates its ability to induce immune cell activation in both assays pointing to a key role for FcγR in delivering the ADC to the immune cell. ADC concentrations are based on payload.

FcγR Mediated Delivery of STING Agonist ADC to Immune Cells Induces Robust Killing of Cancer Cells by PBMCs



Fluorescent Red Nuclear Protein (FRNP)-expressing cancer cells are co-cultured with PBMCs and plates scanned in an IncuCyte Zoom live cell imaging instrument. The number of red objects (cancer cells) is quantified and normalized to T=0 hour for each well.

Figure 4. Targeted ADC induces robust killing of cancer cells by PBMCs. A) Cancer cell killing assay in co-culture with PBMCs. **B)** Targeted ADC at 100x lower concentration than free payload causes robust cancer cell killing by PBMCs. Control ADC did not affect cancer cell viability at the highest concentration used. No cancer cell death observed in mono-cultures. Similar results were obtained using PBMCs from several different donors. **C)** Live images depicting killing of cancer cells as represented by red objects (cancer cells) at the 40 hour timepoint.

Single Doses of Targeted STING Agonist ADCs Outperform IV STING Agonist and Elicit Potent Anti-tumor Immune Responses

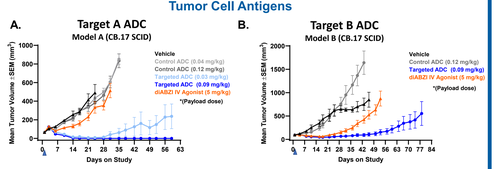


Figure 5. Tumor targeted STING agonist ADCs induce durable and complete tumor regressions in xenograft mouse models at 50x lower doses by payload than the IV administered diABZI STING agonist[2]. A) Single doses of Target A ADC administered intravenously resulted in tumor regression in a Target A expressing xenograft model while control ADC at similar payload doses and the diABZI IV agonist at ~50x higher dose had no significant effect on tumor growth inhibition. **B)** Similarly, single dose of Target B ADC resulted in tumor growth inhibition superior to that obtained with the diABZI IV agonist at ~50x lower payload dose. Control ADC had no significant effect on tumor growth inhibition in this model. All ADCs were well tolerated at the indicated doses.

Single IV Doses of Tumor Targeted STING Agonist ADCs Induce Durable and Complete Tumor Regressions in Syngeneic Mouse Models

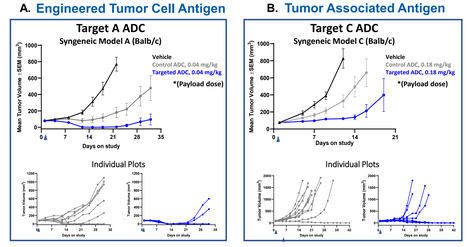
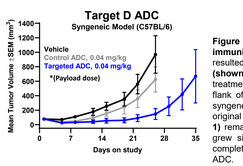


Figure 6. Tumor targeted STING agonist ADCs induce potent anti-tumor immune responses in syngeneic mouse models. A) Single dose of Target A ADC administered intravenously resulted in complete tumor regressions in 8/10 animals in a mouse model engineered to express Target A. Control, non-targeted ADC is significantly differentiated from the targeted ADC indicting tumor targeting. **B)** Similarly, single dose of STING agonist ADC targeted to a tumor associated antigen resulted in durable tumor regressions in 6/10 animals and significant differentiation from the control ADC. All ADCs were well tolerated at the indicated doses.

Sustained Tumor Regressions and Induction of Immunological Memory After Single IV Dose of Targeted STING Agonist ADC

A. Complete & Durable Tumor Regressions by Tumor Associated Targeted STING ADCs



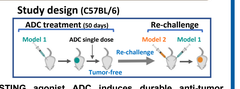
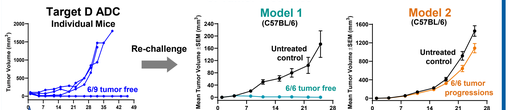


Figure 7. STING agonist ADC induces durable anti-tumor immunity in a syngeneic model. A) Single dose of Target D ADC resulted in complete, durable tumor regressions in 6/9 animals **(shown in B)**. **B)** Six tumor free mice from the targeted ADC treatment were re-challenged with the original tumor on the opposite flank of the original challenge **(Model 1)** and a different C57BL/6 syngeneic model **(Model 2)** was implanted on the same flank of the original challenge. Flanks implanted with the original model **(Model 1)** remained tumor free, while tumors of the new model **(Model 2)** grew similarly to the untreated control, indicating tumor specific, complete immunity to tumor re-challenge mediated by STING agonist ADC.

B. Induction of Durable Immunological Memory Following Re-Challenge of Tumor Free Mice



Doses of STING ADC that Result in Complete Tumor Regressions Induce Significantly Lower Levels of Serum Cytokines than the IV STING Agonist

A. Analysis of Serum Cytokines by Milliplex Immunoassay

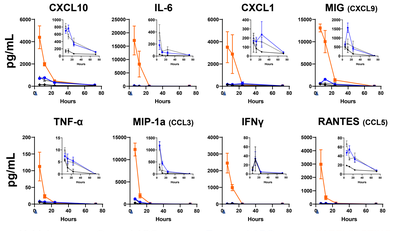
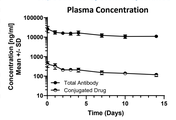


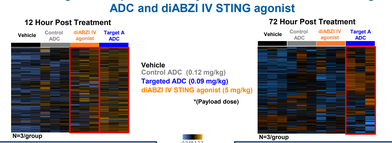
Figure 8. STING agonist ADC induces significantly lower levels of serum cytokines than the diABZI STING agonist administered intravenously. A) Luminex analysis of systemic cytokines in serum samples of tumor bearing mice treated with a single dose of Target A ADC or control ADC and the diABZI IV STING agonist (doses equivalent to those in efficacy study in **Fig. 5A**). Inserts in each plot represent serum cytokine levels of vehicle, targeted and control ADCs on a reduced scale from that of the diABZI IV agonist.
Systemically delivered diABZI STING agonist induced 6-100x higher systemic cytokines than targeted STING agonist ADC despite the lack of sustained anti-tumor activity **(Fig. 5A).** In contrast, extended plasma exposure of Target A STING ADC **(Fig. 8B)** did not result in elevated levels of serum cytokines at a dose equivalent to the one resulting in potent anti-tumor activity in the same model **(Fig 5A).**

B. Extended plasma exposure to STING ADC does not result in extended exposure to systemic cytokines



Sustained Upregulation of Immune Pathway Genes in Tumors Treated with STING Agonist ADC but not with IV STING Agonist

NanoString Gene Expression Analysis of Tumors Treated with Target A ADC and diABZI IV STING agonist



Immune pathway genes are significantly upregulated in tumors treated with targeted ADC and IV STING agonist but not in control ADC or vehicle 12 hours post treatment

Sustained upregulation of immune pathway genes 72 hours post treatment in targeted ADC treatment group only

Figure 9. Sustained STING mediated upregulation of immune pathway genes with targeted STING agonist ADC but not with diABZI IV STING agonist at 50x higher payload dose. Tumor-bearing mice were intravenously injected with a single dose of either the targeted ADC, control ADC, or diABZI IV STING agonist at indicated doses, and tumors were harvested at either 12 or 72 hours and processed into FFPE samples. RNA was extracted and subjected to NanoString analysis. In contrast to data shown in **Fig. 8A** where the diABZI IV STING agonist is shown to induce high levels of inflammatory serum cytokines, tumor cytokines are only transiently induced with this treatment at levels similar to the targeted ADC. Clustering of the genes for the 12 and 72 hour heat maps were done separately.

Targeted but Not Non-Targeted STING Agonist ADC Activates STING Pathway Genes in Tumors

qPCR Analysis of FFPE Tumors Treated with Target A ADC

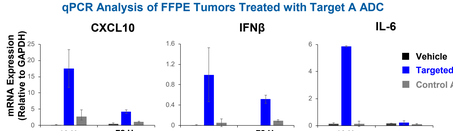


Figure 10. Targeted STING agonist ADC activates STING pathway in tumors significantly better than non-targeted control ADC. Methods for in vivo treatment of tumors and sample processing are similar to those in **Fig 9.** RNA was extracted from FFPE sections and qPCR analysis performed for CXCL10, IFNβ, and IL-6 mRNA expression. GAPDH was used as a reference gene. Cytokine and interferon gene expression is significantly higher in tumors treated with targeted ADC than with control, non-targeted ADC, in contrast to serum cytokines **(Fig. 8A, inserts)** where both targeted and non-targeted ADCs are shown to induce similar levels of circulating cytokines.

Targeted but Not Non-Targeted STING Agonist ADC Induces Marked Immune Cell Infiltration in Tumors

CD45 Immunohistochemistry Staining of FFPE Tumor Tissue

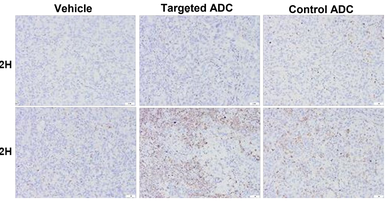


Figure 11. Targeted STING agonist ADC leads to significantly higher CD45+ immune cell infiltration compared to non-targeted control ADC. Tumor-bearing mice were intravenously injected with a single dose of Target A ADC (0.09 mg/kg payload) or control ADC (0.12 mg/kg payload) and tumors harvested at 12 and 72 hours and processed into FFPE samples. CD45 immunohistochemistry staining was performed on the FFPE sections. IHC staining shows significant infiltration of CD45+ immune cells 72 hours post treatment with targeted ADC, with minimal staining observed for control ADC at the same timepoint.

Tolerability and Drug Exposure of STING Agonist ADC in Non-Human Primates

Study design
- Repeat dose every 3 weeks (q3w x 2)
- Assessments included clinical pathology, toxicokinetics, serum cytokines, immunophenotyping (T-B-NK), and histopathology

Clinical Observations
- No treatment related clinical signs and no notable changes in bodyweight or body temperature at doses that resulted in durable and complete regressions in mouse models

Toxicokinetics
- Dose dependent, plasma exposure suitable for drug development
- Minimal free payload detected in plasma confirming high stability of ADCs in circulation

Serum Cytokines
- Transient, dose dependent increase of 5 cytokines/chemokines out of 24 tested (CXCL10, IL-6, MCP1, IL-1ra, MIP1b)

Histopathology
- No adverse histopathology findings

Summary
- Immunosynthen ADC was well tolerated with suitable exposure, without adverse clinical observations or histopathology findings, and only transient increases in a subset of cytokines

CONCLUSIONS

We have demonstrated:

- ❖ Generation of STING agonist ADCs with desirable physicochemical properties
- ❖ Over 100-fold increased potency relative to free agonist, in vitro
- ❖ Highly efficient mechanism of ADC mediated delivery of STING agonist to immune cells and resulting anti-tumor immune responses requiring antigen and FcγR binding
- ❖ Durable, complete tumor regressions after a single IV administration of STING agonist ADCs, significantly differentiated from anti-tumor activity of diABZI IV STING agonist
- ❖ Potent ADC mediated tumor regressions leading to durable immunological memory in an immune competent model
- ❖ Significantly lower induction of serum cytokines relative to diABZI IV agonist and sustained expression of tumor-localized immune pathway genes
- ❖ Efficacious doses of STING ADC are well tolerated in NHP with suitable exposure and excellent ADC plasma stability

Together these data suggest that STING agonist ADC may confer an improved therapeutic index over IV STING agonist, and ADC-mediated systemic delivery of STING agonist activates the immune response locally.

References

1. Corrales et al. ***JCI*** 2016, 126: 2404-2411
2. Ramanjulu et al. ***Nature*** 2018, 564: 438-443
Image was created with BioRender.com

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