Tumor cell-intrinsic STING pathway is activated in the presence of cues from immune cells and contributes to the anti-tumor activity of tumor cell-targeted STING agonist antibody-drug conjugates

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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 allow systemic administration while stimulating the innate immunity in a targeted manner. We have previously demonstrated that targeted delivery of a STING agonist with an ADC induces robust anti-tumor immune responses.

Herein we investigated the mechanism of action of tumor cell-targeted STING agonist ADCs. We evaluated STING pathway activation and antitumor activity elicited by ADCs harboring either wild type (*wt*) or mutant Fc deficient in Fcγ receptor (FcγR) binding in *wt* or STING knockout (*ko*) cancer cell mono-cultures, immune cell co-cultures, and in *in vivo* tumor models. Consistent with previous reports, the majority of cancer cell lines tested failed to induce STING pathway following STING agonist payload treatment in mono-cultures. In cancer cell:THP1 monocytic cell co-cultures, tumor-targeted STING agonist ADCs with *wt* Fc exhibited robust STING activation, whereas Fc-mutant ADCs or non-targeted control ADCs had minimal activity. Similar results were obtained when THP1 cells were treated in plates coated with target antigen without cancer cells, demonstrating STING activation in THP1 cells following FcγR-mediated uptake of antigen-bound ADCs. Tumor-targeted Fc-*wt* ADCs led to marked induction of STING pathway and cancer cell-killing in cancer cell:PBMC or primary monocyte co-cultures, and complete tumor regressions in *in vivo* tumors. Surprisingly, while at reduced levels relative to the Fc-*wt* ADCs, Fc-mutant ADCs exhibited significant activity in these in vitro and in vivo models, suggesting that tumor cell-intrinsic STING pathway may be activated in the presence of cues from immune cells. Consistently, STING agonist payload treatment in the presence of conditioned media from PBMC and primary monocyte but not from THP1 cultures, led to STING activation in cancer cell mono-cultures. Moreover, Fc-mutant ADCs had diminished activity in STING *ko* cancer cell:PBMC or primary monocyte co-cultures, demonstrating the contribution of tumor cell-intrinsic STING activation to the anti-tumor activity elicited by tumor cell-targeted STING agonist ADCs.

In conclusion, we demonstrated that tumor cell-targeted STING agonist ADCs induce robust anti-tumor activity through mechanisms involving both FcγR and tumor antigen-mediated ADC internalization and subsequent induction of STING pathway in immune cells and tumor cells.

Background

Immunosynthen: STING agonist ADC platform

We have previously reported[1] that we have developed novel STING agonist ADCs:

- ❖ Novel agonist payload optimized for ADC, linker & scaffold designed to maximize therapeutic index
- ❖ Robust *in vivo* activity in **6 targets** in 3 mouse strains, induction of immunological memory
- ❖ Superior activity and tolerability vs free IV agonist
- ❖ Well-tolerated in non-human primates
  - No clinical signs
  - High exposure after repeated administration
  - Transient, modest elevation of 5 serum cytokines (24 tested)
  - No adverse changes in clinical pathology or histopathology
- ❖ On track to nominate 1st Development Candidate

Herein we report data demonstrating the mechanism of action of tumor cell-targeted STING agonist ADCs.

Proposed mechanism of action of tumor cell-targeted STING agonist ADCs

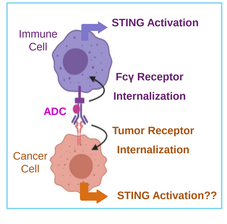


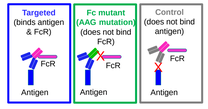
Figure 1. Delivery of a STING agonist into tumor cells and myeloid cells via a tumor cell-targeted ADC.

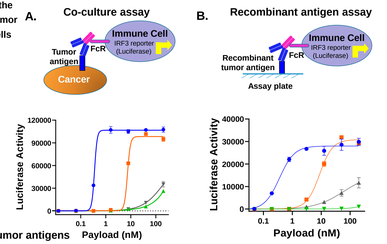
- When bound to their target antigens on tumor cells ADCs can strongly interact and crosslink Fcγ receptors and internalize into immune cells resulting in robust activation of the STING pathway.
- Tumor cell-targeted ADCs are also internalized into tumor cells and can potentially activate tumor intrinsic STING. Although others have reported that STING pathway cannot be activated in tumor cell monocultures[2] tumor-intrinsic STING pathway activity has not been investigated in the presence of immune cells.
- In this study we investigated the contribution of immune cell and tumor cell-intrinsic STING activation to the anti-tumor activity of tumor cell-targeted STING agonist ADCs by utilizing ADCs engineered to deliver STING agonist specifically either into both immune cells and cancer cells, or only into cancer cells in *in vitro* co-cultures of STING wt or ko cancer cells:immune cells and in *in vivo* human tumor xenograft models.

RESULTS

Tumor cell-targeted STING agonist ADCs are internalized into immune cells via Fcγ receptors resulting in robust activation of STING pathway

Three different antibody variants used to confirm the mechanism of Fcγ receptor-mediated delivery of tumor cell-targeted STING agonist ADCs into immune cells





Targeted-ADC-wt
Targeted-ADC-AAG
Control ADC
Free STING agonist

Similar results were obtained with other tumor antigens

Figure 2. Tumor cell-targeted STING agonist ADCs activate STING pathway in THP1 reporter cells in an antigen and Fc-dependent manner. The malignant monocytic cell line (THP1) engineered to express Luciferase from IRF3 reporter was cultured either with cancer cells expressing the target antigen **(A)** or on plates coated with the recombinant tumor antigen **(B)** were treated as indicated for 24 hours and luciferase activity was measured using the Quantiluc reagent (Invivogen). Targeted ADC with wt Fc exhibited ~100x more potency over free agonist and both the control ADC (non-targeted) and the targeted Fc mutant ADC (AAG mutation in the Fc region of the antibody abrogates binding to Fcγ receptors) had minimal activity at the highest doses.

Tumor cell-targeted Fc mutant STING agonist ADCs exhibit significant activity in cancer cell:primary human immune cell co-cultures

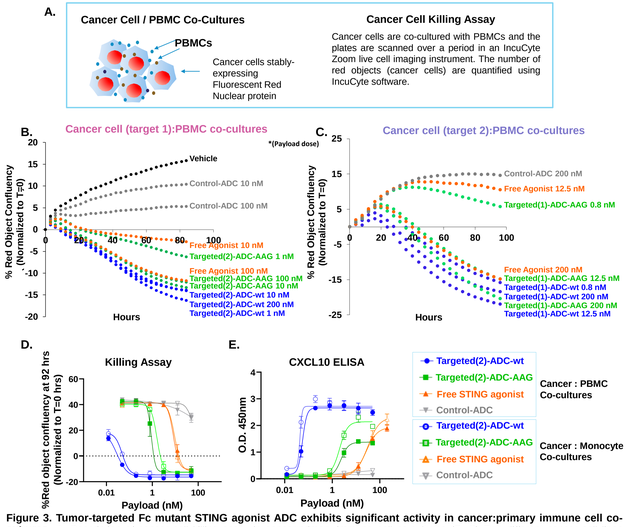


Figure 3. Tumor-targeted Fc mutant STING agonist ADC exhibits significant activity in cancer:primary immune cell co-cultures. A. Description of cancer cell killing assay in cancer:primary immune cell co-cultures **(B, C)** Two different cancer cell lines expressing two different antigens (target 1 (B) or target 2 (C)) were co-cultured with human PBMCs in the presence of the indicated treatments and cancer cell growth was traced over time as described in Fig. 3A. **D.** Cancer cells killing assay as described in (A) was performed to determine the dose responses for the indicated treatments in cancer cell (target 2):PBMC or primary human monocyte co-cultures. **E.** STING pathway activation was determined by analyzing CXCL10 production in supernatants from the sister plates using a human CXCL10 ELISA kit. These data demonstrate the potent induction of STING pathway and robust killing of cancer cells in cancer:primary immune cell co-cultures by targeted ADC with wt Fc. Fc mutant targeted ADC exhibited reduced but significant activity indicating an Fc independent contribution of the targeted ADC in primary human immune cell co-cultures unlike the THP1 (Fig. 2A) co-cultures, suggesting that tumor cells may respond to STING agonism in the presence of cues from primary human immune cells but not from THP1 cells. See Fig. 5 for further discussion.
*Cancer cell lines used in these assays do not respond to STING agonism in monocultures (data not shown).
*Naked targeted antibodies have very minimal impact in cancer cell killing by primary immune cells and CXCL10 induction (data not shown).

Tumor cell-targeted Fc mutant STING agonist ADCs exhibit significant activity in *in vivo* tumor models

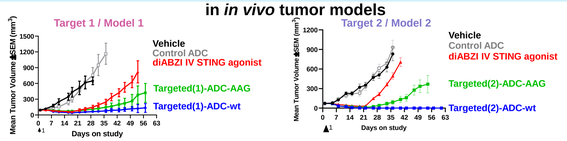
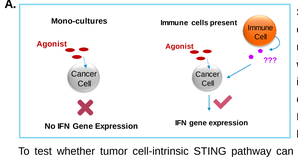


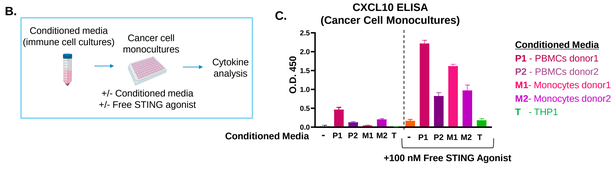
Figure 4. Fc mutant STING agonist ADCs targeting two different tumor antigens exhibit significant activity in two different *in vivo* models. Single doses of intravenously administered ADCs targeting target 1 and target 2 expressed by the tumors led to complete and durable regressions, unlike the diAbzi IV agonist[3], which was administered at a ~50x higher payload dose of the ADCs. Non-targeted control ADC showed no efficacy in either models. In parallel with cancer cell:primary immune cell co-culture results (Fig. 3) Fc mutant targeted ADCs exhibited lower but significant efficacy relative to the targeted ADCs with wt FC in both models suggesting that the tumor-intrinsic STING activation contributes to the tumor-targeted STING agonist ADC anti-tumor activity *in vivo*.

Tumor cell-intrinsic STING pathway can be activated in the presence of cues from primary human immune cells



Since most cancer cell lines, including the ones used in the current study, do not respond to STING agonism in monocultures, we initially anticipated that the Fc mutant ADC, which does not bind to Fcγ receptors and therefore is only internalized into cancer cells, would have minimal activity in cancer cell:immune cell co-cultures. However, we found that Fc mutant ADC exhibited significant activity in cancer:primary human immune cell co-cultures but not in THP1 co-cultures.

To test whether tumor cell-intrinsic STING pathway can be activated in the presence of factors secreted by primary human immune cells but not by the malignant monocytic THP1 cells we treated cancer cell monocultures with the free STING agonist in the presence of conditioned media collected from the untreated cultures (24-36 hours) of human PBMCs, primary monocytes, and THP1 cells and evaluated STING pathway activation.



D. Cytokine analysis of cancer cell line monocultures treated with free STING agonist +/- PBMC conditioned media

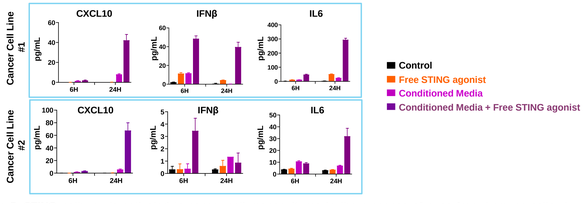
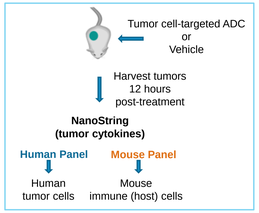


Figure 5. STING pathway is activated in cancer cell monocultures in the presence of cues from immune cells. A. Cartoons depicting the requirement of immune cell-derived factors for STING pathway activation in cancer cells. **B.** Description of the conditioned media experiment. **C.** Supernatants (conditioned media) from untreated cultures of human PBMCs and isolated primary monocytes, and THP1 cells were harvested (spun down to remove immune cells) and added on cancer cell monocultures followed by free STING agonist treatment (100 nM). After 24 hours STING pathway activation was determined by human CXCL10 ELISA assay. STING agonist treatment led to potent activation of STING pathway in cancer cell monocultures only in the presence of conditioned media from PBMCs and monocytes but not from the THP1 cells supporting the hypothesis that STING pathway can be activated in cancer cells in the presence of cues from primary immune cells. **D.** Activation of STING pathway in cancer cell monocultures in the presence of conditioned media from human PBMCs was demonstrated in two other cancer cell lines by analysis of CXCL10, IFNβ, and IL6 cytokine production using a multiplexed Luminex assay. Note that the free STING agonist treatment of cancer cell monocultures did not lead to STING pathway activation in the absence of immune cell-conditioned media (orange bars), which is consistent with the literature.

Tumor cell-targeted STING agonist ADC induces both human and mouse STING pathway genes in human tumor xenografts in SCID mice



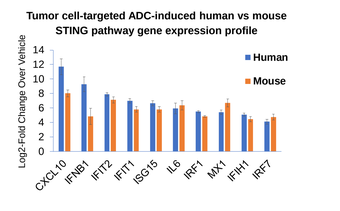


Figure 6. NanoString analysis of human tumor xenografts from SCID mice treated with tumor cell-targeted ADC using either human or mouse gene panels. Tumor-bearing CB.17 SCID mice were injected (IV) with a single dose of tumor cell-targeted STING agonist ADC or vehicle (as in Fig. 4). Tumors were harvested 12 hours later and processed into FFPE. RNA was extracted and analyzed by NanoString using the human or mouse pan-cancer immune gene expression panels. Targeted ADC treatment led to marked induction of both mouse and human STING pathway genes, suggesting that the tumor-targeted STING agonist ADCs may induce tumor-intrinsic STING pathway activation in tumors *in vivo.*

CRISPR knock out of STING in tumor cells confirms the contribution of tumor cell-intrinsic STING to the tumor cell-targeted STING agonist ADC activity

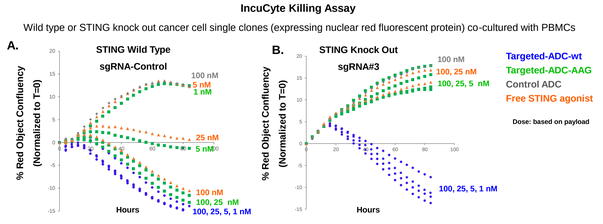


Figure 7. Tumor cell-targeted Fc mutant ADC activity is diminished in STING knock out cancer:PBMC co-cultures. STING wt **(A)** and ko **(B)** cancer cells were co-cultured with PBMCs and cancer cell killing activity of the targeted Fc wt or Fc mutant ADCs, free agonist, and control ADC were assessed by IncuCyte analysis as described in Fig. 3A. Consistent with the results shown in Fig. 3B and 3C, targeted ADC with wt Fc induced significant killing of both STING wt and ko cancer cells in PBMC co-cultures, whereas Fc mutant targeted ADC induced significant killing of only STING wt cells but had no activity in STING ko cancer cell:PBMC co-cultures. Interestingly the free agonist activity was also significantly reduced in the STING ko cancer cell:PBMC co-cultures. Similar results were obtained with STING ko cancer cells generated with two other sgRNAs (2 clones each, data not shown). These data demonstrate the contribution of tumor-intrinsic STING pathway activation to the anti-tumor activity of the tumor cell-targeted STING agonist ADCs in *in vitro* co-cultures of human cancer cell:primary immune cell co-cultures.

CONCLUSIONS

The Immunosynthen ADC platform enables tumor-targeted delivery of a STING agonist with improved efficacy and tolerability over a free IV STING agonist.

In this study we have demonstrated:

- ❖ Tumor antigen-dependent activation of STING pathway in immune cells via Fcγ receptor-mediated internalization of tumor cell-targeted STING agonist ADCs.
- ❖ Tumor cell-intrinsic STING pathway can be activated in the presence of cues from immune cells.
- ❖ Anti-tumor activity of STING agonist ADCs involves activation of STING pathway in both immune cells and tumor cells

The ability of Immunosynthen-based ADCs to activate STING in tumor cells as well as in immune cells may represent a significant therapeutic advantage of targeting the STING pathway.

References:

1. Bukhalid et al. AACR 2020 Abstract #6706
2. Flood et al. Immunological Reviews 2019, 290:24-38
3. Ramanjulu et al. Nature 2018, 564:439-443