Development of a STING Agonist-producing Synthetic Biotic™ Medicine to Activate Innate and Adaptive Immunity and Drive Antitumor Immune Responses

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ABSTRACT

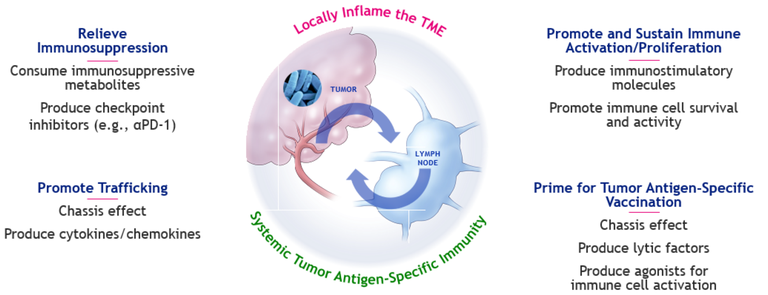
Background: Engagement of both the innate and adaptive arms of the immune system is critical to generate an efficacious anti-tumor immune response. Recent studies demonstrate that activation of the stimulator of interferon genes (STING) pathway plays an essential role in initiating anti-tumor immunity through activation of antigen presenting cells (APCs), production of type I interferon and subsequent T cell priming and tumor-specific T-cell-responses. Bacteria may provide an ideal mechanism for STING activation as they can be deployed within the tumor microenvironment (TME), are engulfed by APCs and activate parallel pathways of innate immunity that may potentiate the interferon response.

Methods: Using synthetic biology we introduced an anaerobically inducible di-nucleotide cyclase gene into our non-pathogenic chassis, *E. coli* Nissle (EcN), to generate a bacterial strain, SYNB1891, capable of efficient production of the STING agonist cyclic-di-AMP (CDA) in response to the hypoxic TME. We then employed a suite of cell-based assays and mouse tumor models to evaluate the activity of SYNB1891 in vitro and in vivo.

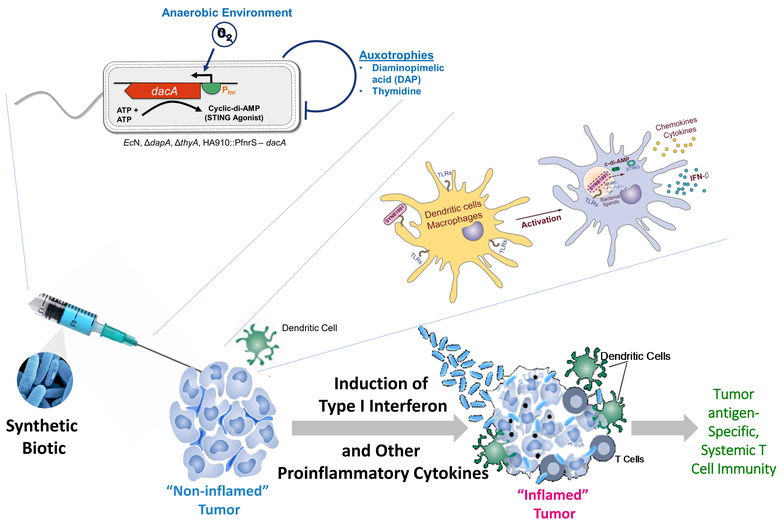
Results: In *in vitro* assays, SYNB1891 generated high levels of CDA and triggered expression of IFNβ when co-cultured with both mouse and human APCs. When compared to naked CDA, we observed that SYNB1891 elicited greater induction of IFNβ in a THP1 luciferase reporter assay and human APCs. In syngeneic tumor-bearing mice, intra-tumoral administration of SYNB1891 resulted in dose-dependent levels of CDA and IFNβ at early time points, as well as other pro-inflammatory cytokines such as IL-6 and GM-CSF. These pharmacodynamic changes correlated with robust, dose-dependent anti-tumor responses and complete tumor regressions. Importantly, we have demonstrated that mice experiencing complete regressions develop systemic immunity and become protected to further challenge with tumor cells.

Conclusions: Taken together, these results demonstrate that a Synthetic Biotic medicine designed to specifically deliver STING agonist locally within the TME leads to significant type I interferon production in the tumor, anti-tumor activity, systemic immunity and long-term immunological memory in preclinical models. Moreover, the ability of our platform to engage multiple innate immune pathways simultaneously further supports the development of Synthetic Biotic medicines for cancer-immunotherapy in humans.

Synlogic Vision for Immuno-Oncology Platform:
Rational Design of Key Immunostimulatory Mechanisms in a Bacterial Chassis



Generation of Synthetic Biotics for Activation of Innate and Adaptive Immunity
Clinical Candidate SYNB1891 "DUAL INNATE IMMUNE ACTIVATOR"



The so called "non-inflamed" or "cold tumors represent a high unmet medical need. At Synlogic we envision developing engineered bacteria or Synthetic Biotic medicines that will engage both arms of the immune system and turn "cold" tumors "hot".

The STING agonist-producing strain, SYNB1891, was generated by integrating the di-adenylate cyclase gene, *dacA,* from the bacterium *Listeria monocytogenes* under the regulatory control of a hypoxia inducible promoter, P_{fnr}, into the genome of the probiotic *E. coli* strain *E. coli Nissle.* In addition, auxotrophies comprising the deletion of genes involved in diaminopimelic acid and thymidine (ΔdapA/ΔthyA) synthesis, were also introduced as a dual biosafety feature.

Intra-tumoral delivery of SYNB1891 into the TME results in robust induction of Type I Interferon through the dual activation of the STING pathway and pattern recognition receptors, leading to subsequent induction of innate and adaptive immune responses and the generation durable systemic anti-tumor immunity.

Table of Synthetic Biotic Strains

Strain	Genetic Content
SYN	Un-engineered Ecoli Nissle:Abx+
SYNB	DAP/Thy dln EcN (no dacA insert):Abx+
SYNB1891-PT1	DAP dln EcN:dacA$_{plasmid}$:FnR-inducible:Abx+
SYNB1891-PT2	DAP/Thy dln EcN:dacA$_{integrated}$:FnR-inducible:Abx+
SYNB1891	DAP/Thy dln EcN:dacA$_{integrated}$:FnR-inducible:Abx-

RESULTS

In Vitro Characterization of SYNB1891 Synthetic Biotic
SYNB1891 Produces Intracellular CDA and Induces a Greater IFN Response Compared to Naked Agonist

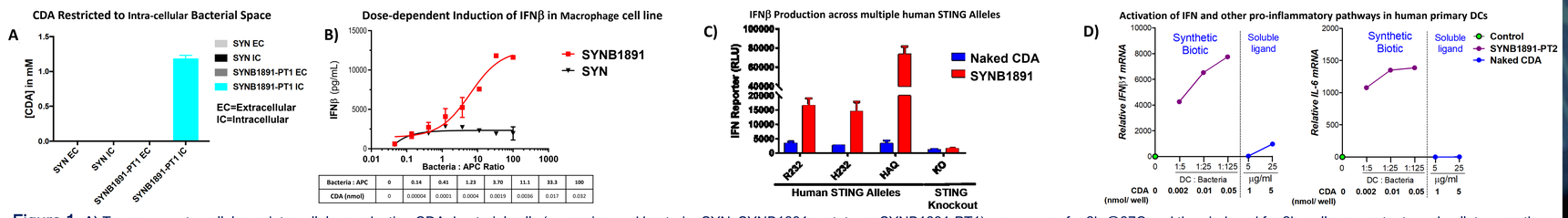


Figure 1. **A)** To assess extracellular vs intracellular production CDA, bacterial cells (un-engineered bacteria=SYN; SYNB1891 prototype=SYNB1891-PT1) were grown for 2h @37C and then induced for 2h, cell supernatants and pellets were then harvested and analyzed by LCMS. **B)** For in vitro pharmacological characterization, CDA activity was measured in un-engineered SYN bacteria and compared to SYNB1891 induced under anaerobic conditions. Cells pellets were harvested and the intracellular CDA concentrations were analyzed by LCMS. For the cell-based activity of SYNB1891 RAW cells were incubated with different MOI of the different strains for 4h and then IFNβ concentrations from cell supernatants were analyzed by Luminex analysis. **C)** To evaluate the activity of SYNB1891 against different human STING alleles, an THP-1 IFN reporter cell assay was used. THP-1 cells containing knock-ins of the different STING alleles or a STING knock-out were incubated with either naked CDA (100 ug) or a ratio of SYNB1891-to-THP-1 where the CDA production was equivalent to 100 ug, and bioluminescence quantified. **D)** Human dendritic cells (DCs) were co-incubated with PBS (Control), different DC : Bacteria ratios (1:5, 1:25 or 1:125) or with different concentrations of soluble naked STING agonist (5 and 25 ug/mL) for 2h @37C and then the DCs were harvested and processed for analysis of IFNβ and IL-6 transcripts.

In Vivo Characterization of SYNB1891 Synthetic Biotic
SYNB1891 Delivers Anti-tumor Activity as a Single Agent

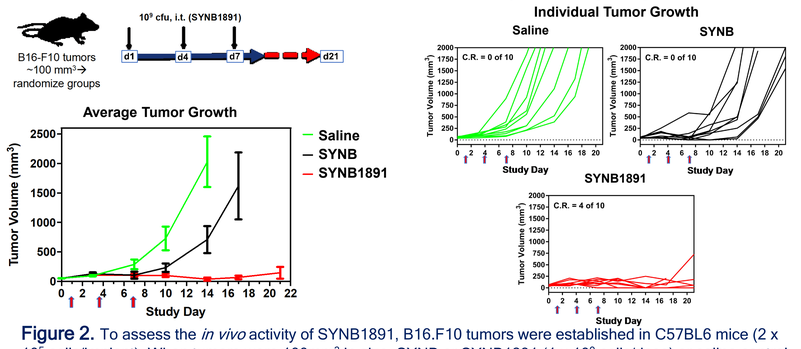


Figure 2. To assess the *in vivo* activity of SYNB1891, B16.F10 tumors were established in C57BL6 mice (2 x 10^5 cells/implant). When tumors were 100mm^3 in size, SYNB or SYNB1891 (1 x 10^9 cells/dose) or saline control were administered as 3 intra-tumoral injections (Q3D x 3) and tumor measurements were taken 2 times/week to determine tumor volumes.

Pharmacologic Characterization of SYNB1891
Administration of SYNB1891 Results in Dose-dependent Efficacy

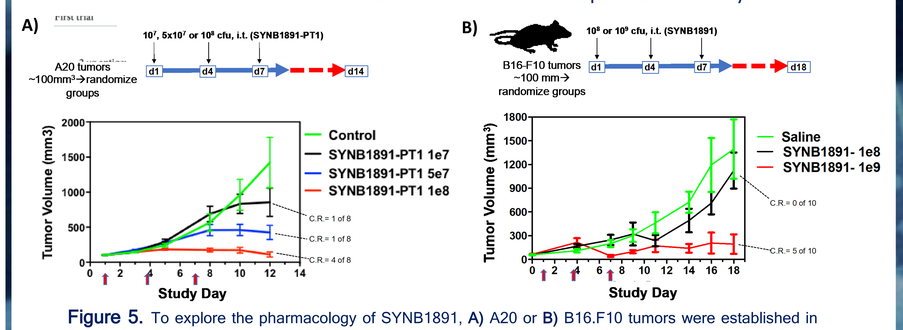


Figure 5. To explore the pharmacology of SYNB1891, **A)** A20 or **B)** B16.F10 tumors were established in BalbC (2 x 10^5 cells/implant) or C57BL6 (2 x 10^5 cells/implant) mice respectively. When tumors were 100mm^3 in size SYNB-PT1 or SYNB1891 was administered intra-tumorally for 3 doses (D1, 4 and 7) at the indicated cells/dose and compared to saline control. Tumor measurements were taken 2 times/week to determine tumor volumes.

Pharmacodynamic Characterization of SYNB1891
Administration of SYNB1891 Results in Dose-dependent Increases in Tumoral CDA, IFNβ and Innate Immune Cytokines

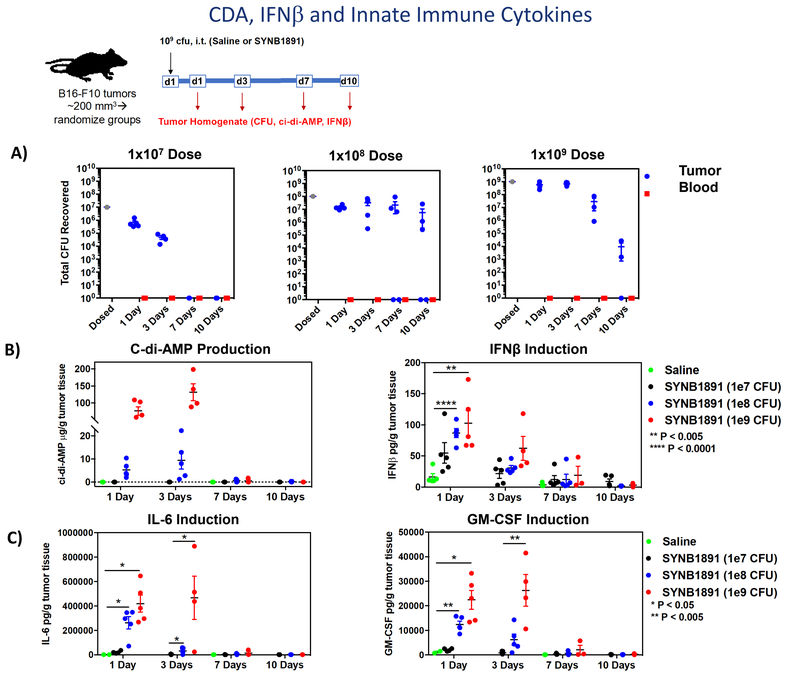


Figure 3. To explore the bacterial kinetics and pharmacodynamics of the SYNB1891 strain, B16.F10 tumors were established in C57BL6 mice (2 x 10^5 cells/implant). When tumors were 200 mm^3 in size, SYNB1891 (1 x 10^7, 1 x 10^8 or 1 x 10^9 cells/dose) or saline control were were administered as a single intra-tumoral injection and tumors were harvested for *ex-vivo* tumoral analysis of **A)** bacterial abundance (CFU), **B)** CDA levels and IFNβ production and **C)** IL-6 and GM-CSF levels.

SYNB1891 Elicits an Inflammatory Gene Signature
SYNB1891 Prototype-treated Tumors Elicits an Inflammation-related Gene Signature

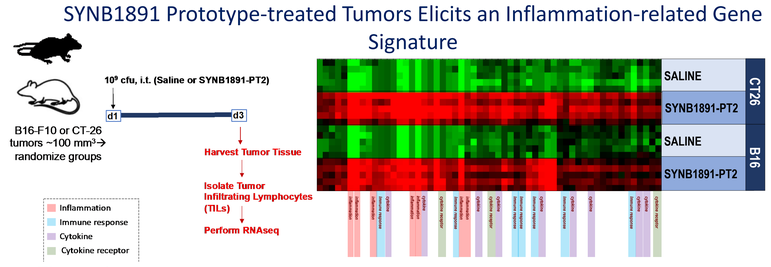


Figure 4. To investigate the transcriptional changes induced by SYNB1891 administration, B16.F10 tumors (2 x 10^5 cells/implant) and CT26 (1 x 10^6 cells/implant) were established in C57BL6 and BalbC mice respectively. When tumors were 100 mm^3 in size SYNB1891-PT2 was administered as 1 intra-tumoral dose (D1) at 1 x 10^9 and tumors were harvested on D3 post dose and processed for TIL isolation and subsequent RNAseq analysis.

Generation of Systemic Immunity
Administration of SYNB1891 Prototype Strain Leads to Systemic Anti-tumor Immunity

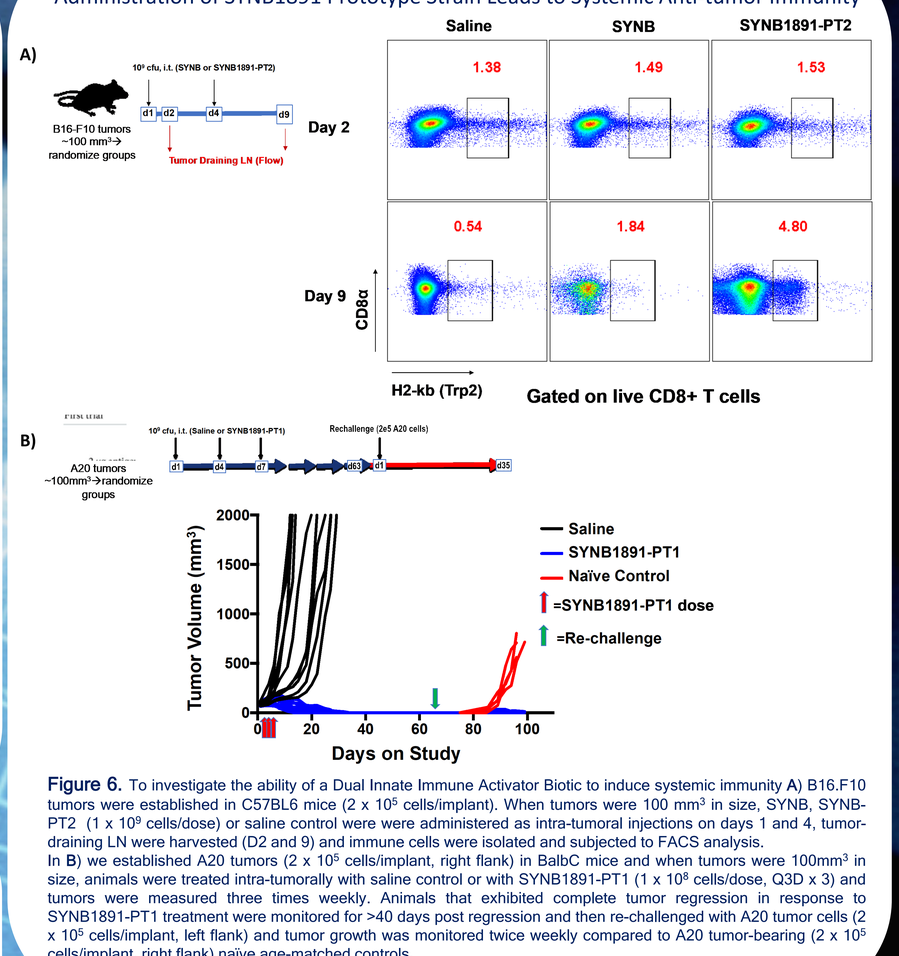


Figure 6. To investigate the ability of a Dual Innate Immune Activator Biotic to induce systemic immunity **A)** B16.F10 tumors were established in C57BL6 mice (2 x 10^5 cells/implant). When tumors were 100 mm^3 in size, SYNB, SYNB-PT2 (1 x 10^9 cells/dose) or saline control were were administered as intra-tumoral injections on days 1 and 4, tumor-draining LN were harvested (D2 and 9) and immune cells were isolated and subjected to FACS analysis.
In **B)** we established A20 tumors (2 x 10^5 cells/implant, right flank) in BalbC mice and when tumors were 100mm^3 in size, animals were treated intra-tumorally with saline control or with SYNB1891-PT1 (1 x 10^8 cells/dose, Q3D x 3) and tumors were measured three times weekly. Animals that exhibited complete tumor regression in response to SYNB1891-PT1 treatment were monitored for >40 days post regression and then re-challenged with A20 tumor cells (2 x 10^5 cells/implant, left flank) and tumor growth was monitored twice weekly compared to A20 tumor-bearing (2 x 10^5 cells/implant, right flank) naïve age-matched controls.

CONCLUSIONS

- We have generated an engineered bacterial strain or Synthetic Biotic medicine, SYNB1891, that is capable of specific and efficient dual activation of innate immunity through engagement of TLRs and delivery of c-di-AMP (CDA) within the TME. We refer to this biotic as a "**Dual Innate Immune Activator**".
- We have demonstrated that administration of SYNB1891 in mouse tumor models results in increased tumoral CDA, IFNβ and innate immune cytokines, dose dependent, single-agent anti-tumor activity and the generation of systemic anti-tumor immunity.
- Based on our pre-clinical results we have declared SYNB1891 our Clinical Development Candidate