# Activation of innate and adaptive immunity via combinatorial immunotherapy using Synthetic Biotic™ Medicines

Daniel Leventhal*∞, Adam Fisher∞, Kip A. West, Anna Sokolovska, Starsha Kolodziej, Ning Li, Chris Plescia, Carey Gallant, Mary Castillo, Paul Miller and Jose M. Lora

Synlogic Inc., Cambridge, MA, 02142; * = presenting author, ∞ = co-authors

ABSTRACT#: LB-131

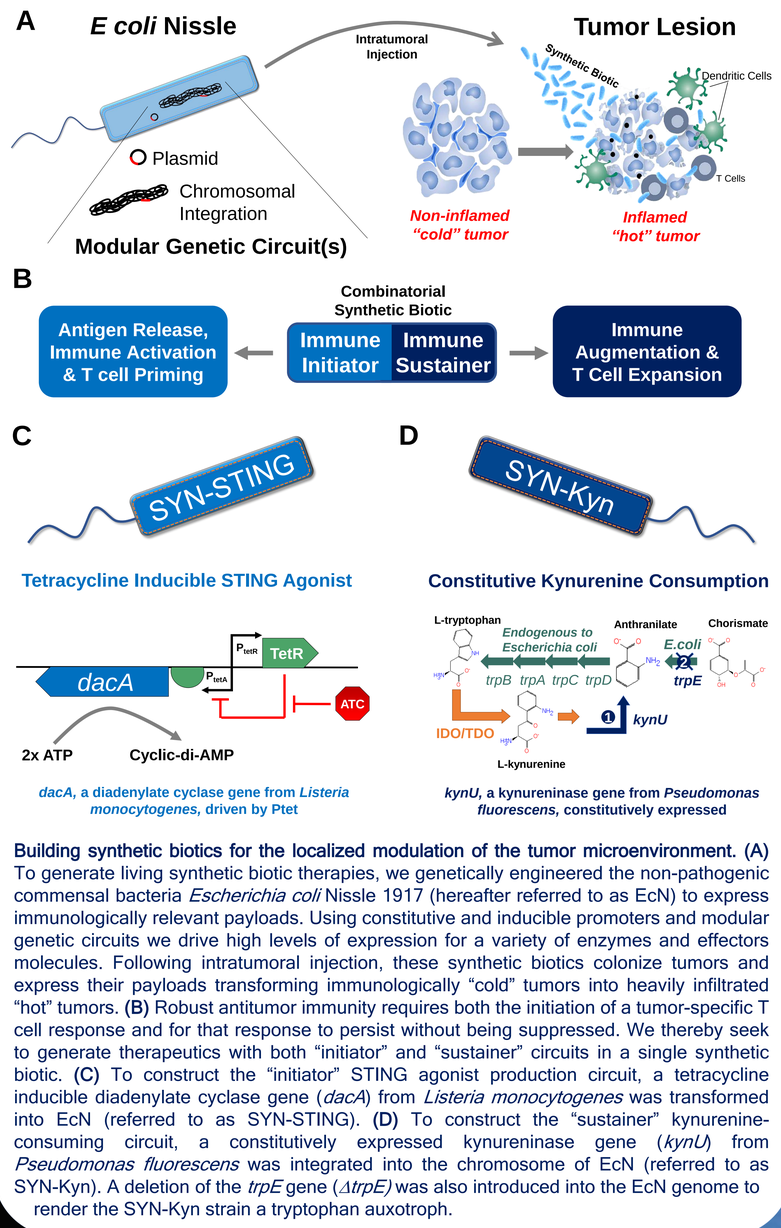
## ABSTRACT

***Background:*** While both T-cell priming and blockade of immune suppression play critical roles in the generation of an efficacious antitumor immune response, most therapies fail to support both processes as a single agent. At Synlogic we are using synthetic biology and engineered bacteria programmed with immuno-modulatory circuits to develop treatments or "Synthetic Biotic Medicines" capable of simultaneously engaging multiple pathways relevant for the treatment of cancer and autoimmunity. Recent studies have demonstrated that activation of the **st**imulator of **in**terferon **g**enes (STING) pathway can play a critical role in the initiation of an antitumor immune response via activation of antigen presenting cells (APCs) and presentation of tumor antigens. Additionally, metabolites derived from biosynthetic pathways, such as conversion of tryptophan into kynurenine by **i**ndoleamine 2,3 **dio**xygenase (IDO), have also been recently appreciated as major components of the immune-suppressive tumor microenvironment which lead to T cell dysfunction and exhaustion. Here we present results showing the development of two circuits, an immune "initiator" STING activating circuit (SYN-STING) and an immune "sustainer" Kynurenine consuming circuit (SYN-Kyn), in engineered strains of *E. coli* Nissle.

***Methods and Results:*** Synthetic biological techniques were employed to generate bacterial strains expressing various protein components capable of (1) generating the STING agonist cyclic-di-AMP (SYN-STING) or (2) actively transporting and metabolizing kynurenine (SYN-Kyn). In *in vitro* biochemical and functional assays, SYN-STING generated high levels of cyclic-di-AMP and triggered the expression of IFNβ1 and IL-6 upon co-culture with APCs, while SYN-Kyn actively depleted test media containing kynurenine at levels 20-fold higher than those found in tumors of cancer patients. In mice bearing subcutaneous syngeneic tumors the intratumoral administration of SYN-STING resulted in an early rise of innate cytokines which several days later shifted towards molecules indicative of an effector-T cell response. Additionally, the administration of a single dose of SYN-Kyn led to significant decreases in tumor kynurenine levels atleast 72 hours post dose. Finally, administration of either SYN-STING as a single agent, or SYN-Kyn in combination with anti-CTLA4 antibodies in tumor-bearing mice led to significant anti-tumor effects.

***Conclusions:*** Taken together, these results demonstrate that the use of synthetic biology to engineer bacteria is a viable approach to deliver profound efficacy in experimental models of cancer. The data supports the further development of Synthetic Biotic Medicines capable of locally targeting multiple immune pathways as single immuno-oncology therapeutic agents in patients with cancer.

## Constructing Synthetic Biotics to Modulate the Tumor Microenvironment




**Building synthetic biotics for the localized modulation of the tumor microenvironment. (A)** To generate living synthetic biotic therapies, we genetically engineered the non-pathogenic commensal bacteria *Escherichia coli* Nissle 1917 (hereafter referred to as EcN) to express immunologically relevant payloads. Using constitutive and inducible promoters and modular genetic circuits we drive high levels of expression for a variety of enzymes and effectors molecules. Following intratumoral injection, these synthetic biotics colonize tumors and express their payloads transforming immunologically "cold" tumors into heavily infiltrated "hot" tumors. **(B)** Robust antitumor immunity requires both the initiation of a tumor-specific T cell response and for that response to persist without being suppressed. We thereby seek to generate therapeutics with both "initiator" and "sustainer" circuits in a single synthetic biotic. **(C)** To construct the "initiator" STING agonist production circuit, a tetracycline inducible diadenylate cyclase gene (*dacA*) from *Listeria monocytogenes* was transformed into EcN (referred to as SYN-STING). **(D)** To construct the "sustainer" kynurenine-consuming circuit, a constitutively expressed kynureninase gene (*kynU*) from *Pseudomonas fluorescens* was integrated into the chromosome of EcN (referred to as SYN-Kyn). A deletion of the *trpE* gene (*ΔtrpE)* was also introduced into the EcN genome to render the SYN-Kyn strain a tryptophan auxotroph.

## RESULTS

### Activity of SYN-STING and SYN-Kyn strains *in vitro*

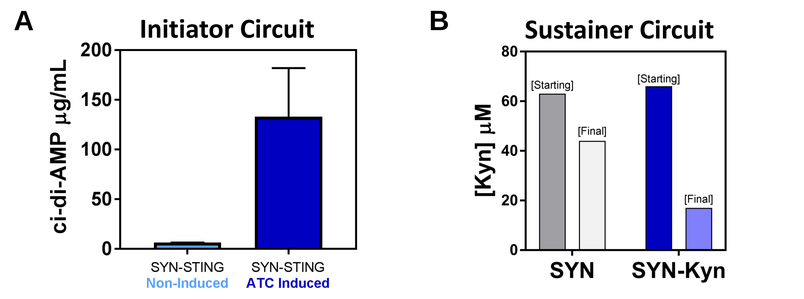



**Figure 1:** ***In vitro*** **STING agonist (ci-di-AMP) production and kynurenine (Kyn) consumption. (A)** SYN-STING was exposed to 200 ng/mL anhydrous tetracycline (aTC) for 4 hours. Levels of intracellular ci-di-AMP were then analyzed via LCMS of bacterial pellet samples. **(B)** SYN (non-engineered bacteria) or SYN-Kyn were incubated with an initial concentration of ~80 uM Kyn at 37°C for 2.5 hours and then samples were analyzed via LCMS to assess kynurenine consumption.

### STING pathway activation in antigen presenting cells

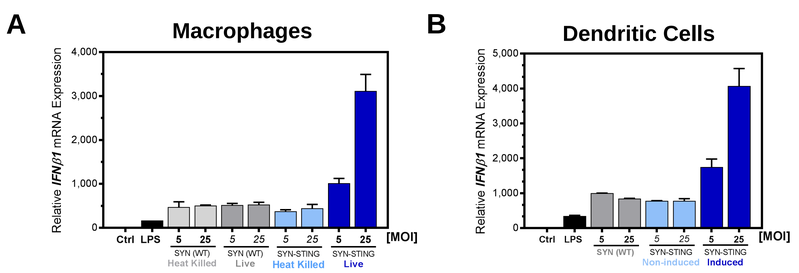



**Figure 2:** ***In vitro*** **analysis of SYN-STING activity following co-culture with macrophages and dendritic cells (DCs).** To assess the ability of SYN-STING to activate the STING pathway in antigen presenting cell populations we co-cultured bacteria at various multiplicities of infection (MOI) with 0.5x10$^6$ RAW 264.7 cells (immortalized murine macrophage cell line) or murine bone marrow-derived DCs. Co-cultures were incubated for 2 hours and mRNA was harvested to measure IFNβ1 gene induction via quantitative PCR. (A) Mean IFNβ1 gene induction of RAW 264.7 cells. Heat-killed bacteria were generated at 60°C for 30 min. (B) Mean IFNβ1 gene induction of DCs. Ctrl = control PBS; LPS = 100 ng/mL lipopolysaccharide. All signals normalized to PBS treated controls.

### Treatment with SYN-STING results in rapid tumor control and activation of innate and adaptive immunity

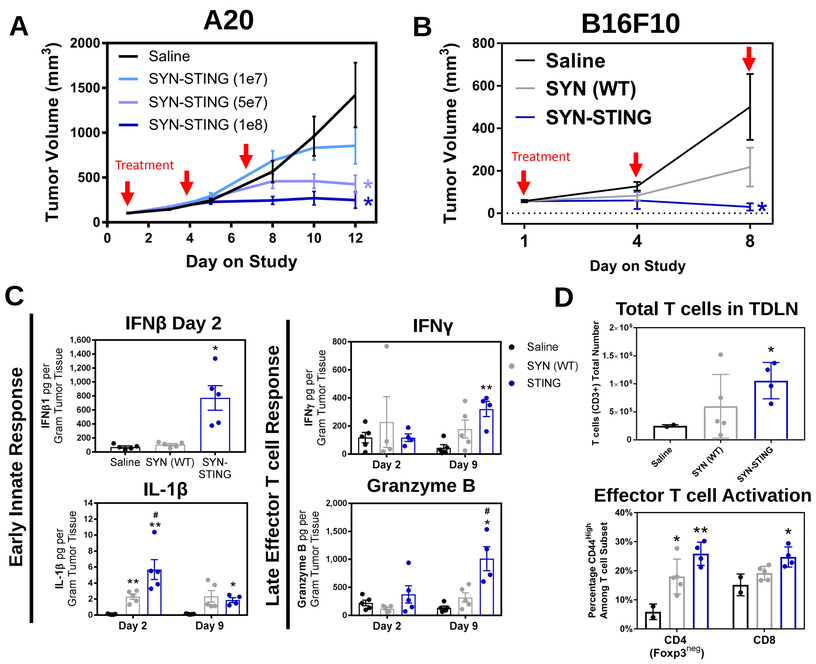



**Figure 3: Impact on tumor growth, cytokine expression and T cell response following intratumoral administration of A20 and B16F10 tumors with SYN-STING.** A20 and B16F10 tumors (~40-80mm$^3$) received three doses of saline or bacteria (1x10$^7$, 5x10$^7$ or 1x10$^8$ CFUs for A20 and 1x10$^7$ for B16F10) via intratumoral injection. Four hours later mice received 10 µg aTC via intraperitoneal injection. Average tumor volume with mean and S.E.M. is shown for **(A)** A20 and **(B)** B16F10 tumors. **(C)** Cytokine expression of treated B16F10 tumor homogenates were analyzed by Luminex bead array on days 2 and 9 post treatment initiation. **(D)** T cells taken from tumor draining lymph nodes from B16F10 tumor bearing mice were analyzed 9 days post treatment initiation via flow cytometry using the following markers: Live/Dead, CD3, CD4, CD8, CD44 and Foxp3 (intracellular). Statistical significance determined using unpaired t test (A, B &D) or Holm-Sidak method adjusted for multiple T test (C). Indicated group compared to Saline; * P < 0.05, ** P <0.005. Indicated group compared to SYN (WT); # P < 0.05.

### Tumor colonization and Kyn consumption by SYN-Kyn

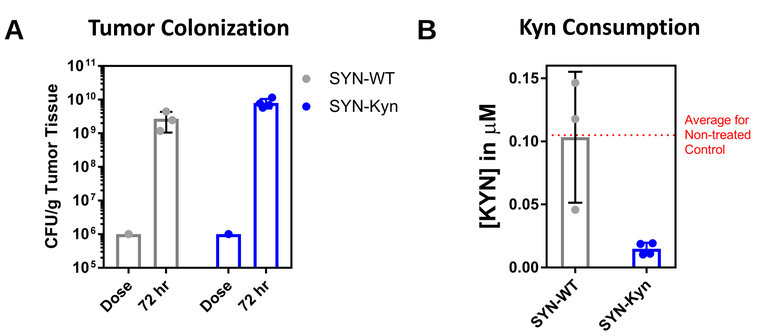



**Figure 4: Analysis of SYN-Kyn tumor colonization and** ***in vivo*** **activity.** Upon reaching ~40-80mm$^3$ B16F10 tumors received 1x10$^6$ CFUs of SYN-WT or SYN-Kyn bacteria via intratumoral injection. 72 hours post-injection tumors were homogenized and colony forming units (CFU) were determined by plating on LB antibiotic selective plates **(A)** or kyn levels were determined by LCMS **(B)**.

### SYN-Kyn plus α-CTLA4 elicits long term tumor control

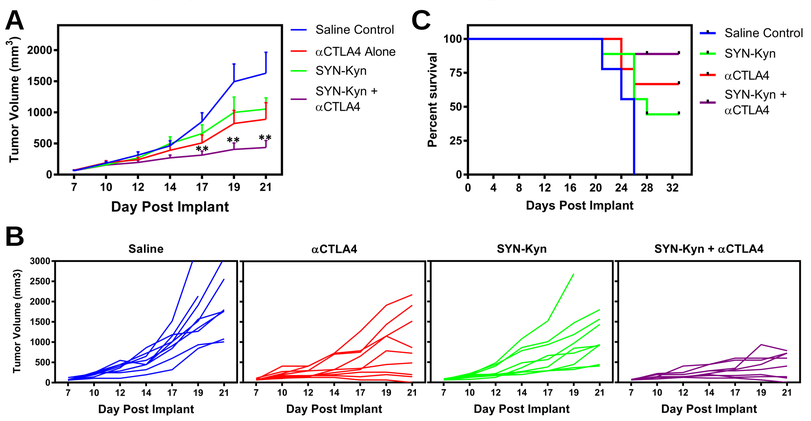



**Figure 5: Therapeutic efficacy of SYN-Kyn treatment in combination with CTLA4 checkpoint blockade in the CT26 tumor model.** Upon reaching ~60-80mm$^3$ in size, CT26 tumor-bearing mice were treated bi-weekly with intratumoral injections of saline control, intraperitoneally with anti-CTLA4 antibody (5 mg/kg), 1x10$^6$ CFUs of SYN-Kyn, or a combination of anti-CTLA4 and SYN-Kyn. **(A)** Average mean tumor volume and S.E.M. **(B)** Individual tumor volumes for each treatment group. **(C)** Percentage of animals remaining on study over time using tumor volume <2000mm$^3$ as a survival surrogate. Statistical significance determined using Holm-Sidak method adjusted for multiple T tests. Indicated group compared to Non-treated or Saline Controls at the indicated time point; ** P <0.005.

### Simultaneous ci-di-AMP production and Kyn consumption by SYN-STING:Kyn

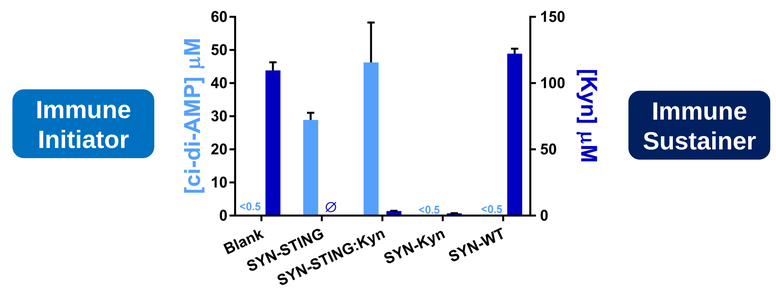



**Figure 6:** ***In vitro*** **analysis of ci-di-AMP production and Kyn consumption for SYN-STING:Kyn.** Bacteria strains containing both circuits were prepared and assays for Kyn consumption or ci-di-AMP production as described in Figure 1 with analytes being measured via LCMS. Kyn concentrations are shown in blue and ci-di-AMP concentrations shown in red for each strain. <0.5 = less than 0.5uM for the indicated analyte; ∅ = analyte concentration not determined.

## SUMMARY AND CONCLUSIONS

Both priming and sustaining antitumor immunity is critical for the efficacy of any immunotherapy. Here we demonstrate:

- The generation of engineered strains of *E. coli* Nissle capable of producing high levels of the STING agonist ci-di-AMP and efficiently metabolizing the immunosuppressive metabolite kynurenine
- *In vitro* SYN-STING produces biologically-relevant levels of ci-di-AMP, activating antigen presenting cells, while SYN-Kyn depletes kynurenine at clinically relevant concentrations
- *In* vivo, intratumoral injection of SYN-STING results in robust efficacy which correlates with an early rise in innate-immune cytokines that later results in T cell activation in tumors and tumor-draining lymph nodes. Similarly, combination of SYN-Kyn with checkpoint inhibition led to robust anti-tumor activity.
- The data presented above suggests that the utilization of Synthetic Biotic medicines is a promising approach to engage multiple immune pathways in the tumor microenvironment, and supports exploring their clinical activity in patients with cancer