A Synthetic Biology Approach for the Treatment of Cancer and Inflammation

Daniel Leventhal, Chris Plescia, Ning Li, Adam Fisher, Kip A. West, Starsha Kolodziej, Rudy Christmas, Anna Sokolovska, Megan McPhillips, Carey Gallant, Michael James, Mary Castillo, Paul Miller and Jose M. Lora, Synlogic Inc., Cambridge, MA, 02139.

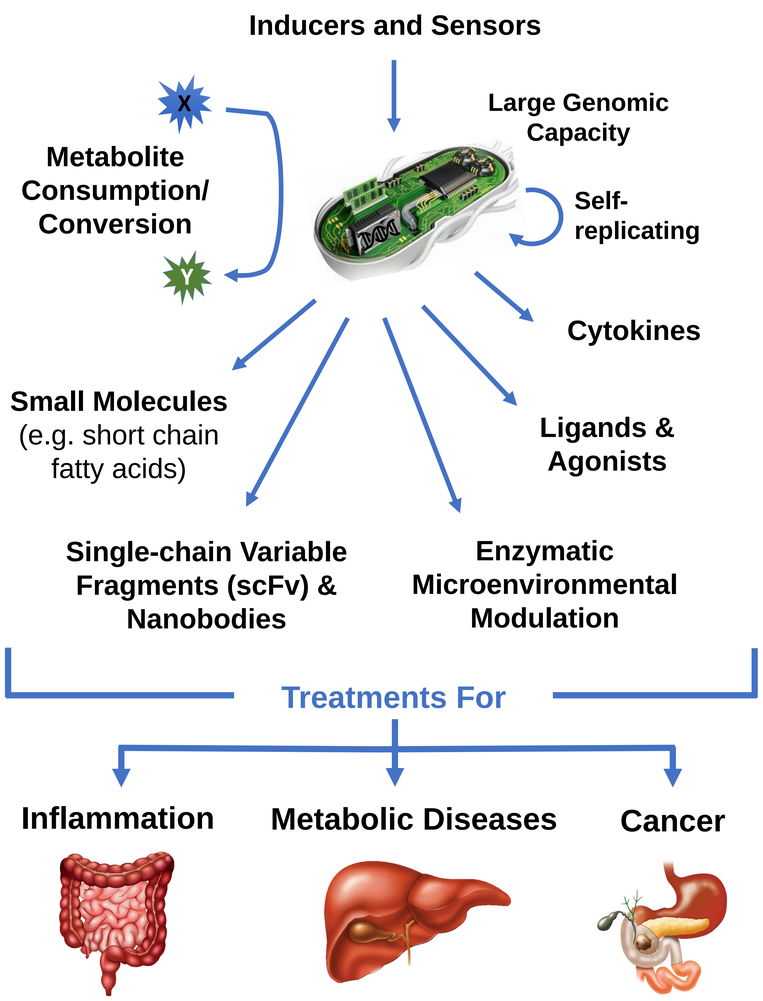
ABSTRACT

At Synlogic we apply synthetic biology to non-pathogenic bacteria (*E. coli* Nissle) to develop "Synthetic Biotic Medicines" capable of manipulating multiple pathways relevant for the treatment of cancer and autoimmunity. Our synthetic biology platform allows us to design bacterial strains capable of executing metabolic conversions (production or consumption of metabolites), secretion of proteins (chemokines, cytokines, enzymes) and secretion or display of single-chain Fv (scFv) molecules (to interfere with ligand-receptor interactions).

We have applied this synthetic biology platform to modulate immune responses in the context of cancer and inflammation. In cancer we effectively trigger innate and adaptive immune responses by intratumoral expression of a variety of effectors, such as STING agonists, TNFα and IFNγ, and reverse immunosuppression by consumption of suppressive metabolites. These strains show robust anti-tumor activity in B16F10 and CT26 syngeneic mouse models, as single agents or in combination with checkpoint inhibitors. In inflammation, we have successfully built strains that produce an array of immunomodulatory metabolites (such as short chain fatty acids and tryptophan derivatives) as well as immunomodulatory cytokines. Given orally, these strains demonstrate robust modulation of immune cellular subsets and inflammation, both locally in the gut as well as systemically.

Taken together, these results establish our synthetic biology-based platform as a robust system for the localized and sustained delivery of immunological payloads to the tumor microenvironment as well as the gut, and support the development of Synthetic Biotic Medicines as a novel approach to treat immune-mediated diseases, both in cancer and inflammation/autoimmunity.

Synthetic Biotics Medicines: Utilizing *E. Coli* Nissle as a Multifunctional Platform for Treating Human Disease



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).

Engineering Non-pathogenic *E.coli* Nissle for the Treatment of Cancer

E.coli Nissle selectively colonize and persist long term within tumors and can be engineered to respond to various inputs

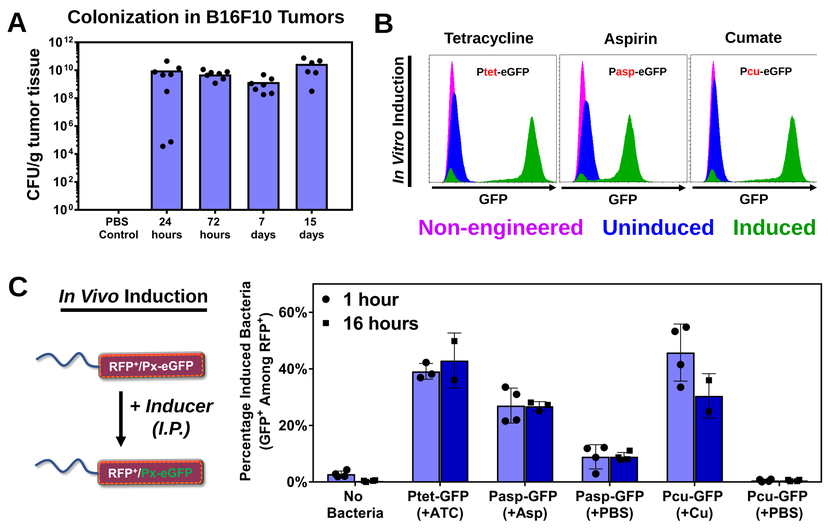


Figure 1: *E.coli* Nissle is a robust engineerable vector for the treatment of cancer. (A) 1e6 CFUs of Nissle was intratumorally administered into established B16F10 tumors and presence of bacteria was measured by colony forming unit assay over time. (**B**) Nissle strains engineered to express eGFP under the indicated promoters were exposed to various inducer agents or conditions and analyzed by flow cytometry. (**C**) Nissel strains constitutively expressing mCherry and engineered to inducably express eGFP as described above were injected in B16F10 tumors. Two days post injection mice were treated (I.P.) with various inducer agents or PBS control and intratumoral bacteria was harvested 1 and 16 hours post treatment. Percentage GFP induction among all RFP+ bacteria was measured by flow.

Consuming immuno-suppressive metabolites from the tumor microenvironment

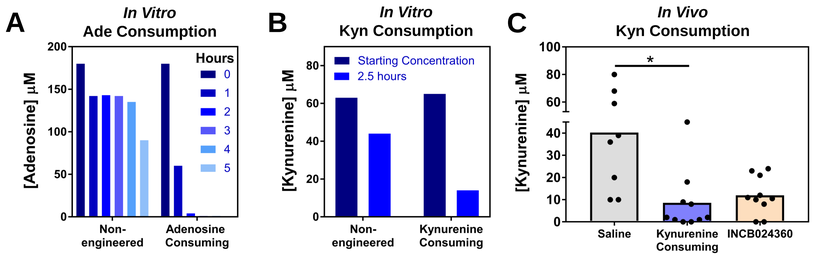


Figure 2: Engineered Nissle can consume the immuno-suppressive metabolites Ade and Kyn. To assess *in vitro* metabolite consumption activity, non-engineered bacteria was compared to a (A) adenosine-consuming strain or the (**B**) kynurenine-consuming strain. Cells were incubated with an initial concentration of 180 uM of Ade or 80 uM Kyn respectively at 37°C and then samples were analyzed via LCMS. (**C**) *In vivo* activity of the Kyn consuming strain was compared to treatment with a bench-mark IDO inhibitor (INCB024360) following I.T. or I.P. administration of CT26 tumor bearing mice, respectively. Statistical significance determined using unpaired t test, * P < 0.05.

Production and secretion of biologically active cytokines (IL-15, IFNγ and TNFα)

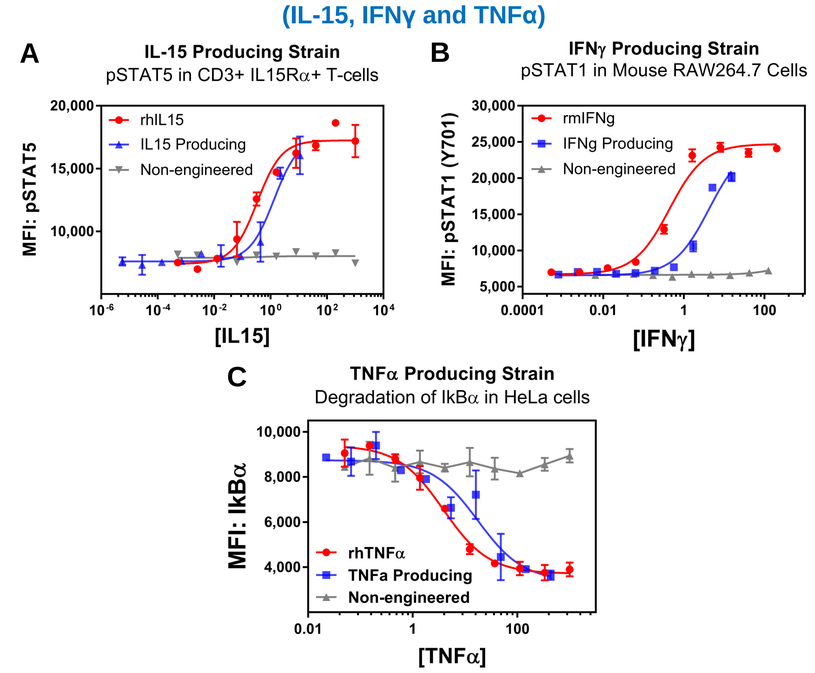


Figure 3: Activity of IL-15, IFNγ and TNFα secreting strains. The *in vitro* activity of various cytokines were evaluated via cell based assay utilizing serial dilutions of bacterial supernatants. (**A**) PHA-L activated human T cells were stimulated for 20 min at 37°C and analyzed for pSTAT5 by flow. (**B**) RAW264.7 cells were stimulated for 15 min at 37°C and analyzed by flow for pSTAT1. (**C**) HeLa cells were stimulated for 10 min at 37°C and analyzed by flow for loss of IkBα.

Production of high levels of intratumoral TNFα and control of tumor growth in CT26 Tumors

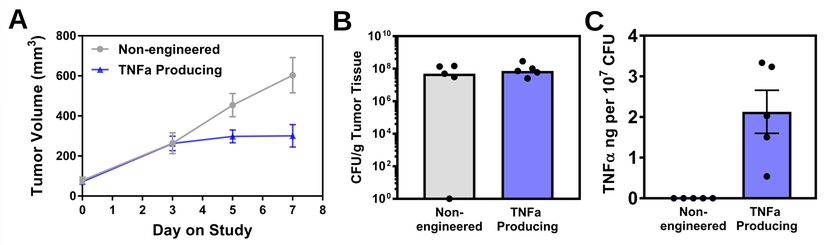


Figure 4: *In Vivo* secretion of TNFα and impact on CT26 tumor growth. 1e7 CFUs of Wildtype or Nissle engineered to secrete TNFα were I.T. administered into established CT26 tumors. Mice were treated with anhydrous tetracycline I.P. on days 1, 4 and 7 to induce expression of TNFα. (**A**) Mean tumor volume is shown for each experimental group. (**B & C**)Tumors were harvested on day 8 and analyzed for bacterial presence by CFU assay and abundance of TNFα by ELISA.

Intratumoral enzymatic conversion of pro-drugs (5-FC → 5-FU)

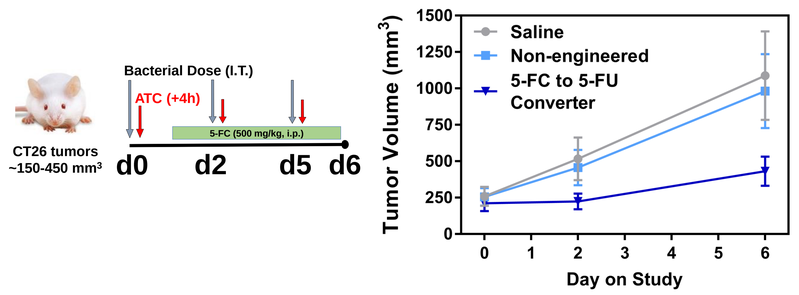


Figure 5: Conversion of the prodrug 5-FC to 5-FU by engineered Nissle. Large (150-450mm^3) CT26 tumors were treated with Nissle expressing cytosine deaminase (CD) under the tetracycline promoter. Mice were treated with ATC 4 hours later to induce expression of CD and starting 24 hours later given daily I.P. doses of 5-FC. Mice were monitored for signs of toxicity and tumor growth.

Engineering an inducible STING agonist (ci-di-AMP) producing circuit in *E.coli* Nissle

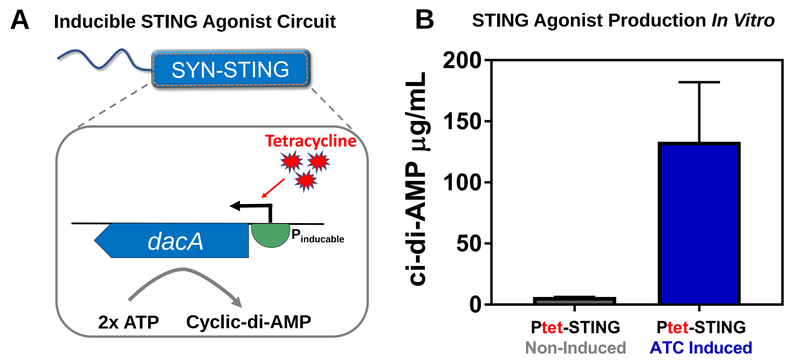


Figure 6: Engineering the inducible expression of ci-di-AMP in EcN. (A) To construct the STING agonist production circuit, a tetracycline (pTet) inducible diadenylate cyclase gene (*dacA*) from *Listeria monocytogenes* was transformed into EcN (referred to as SYN-STING). (**B**) SYN-STING was exposed 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.

High levels of intratumoral ci-di-AMP are detected following SYN-STING treatment which results in tumor control and rejection

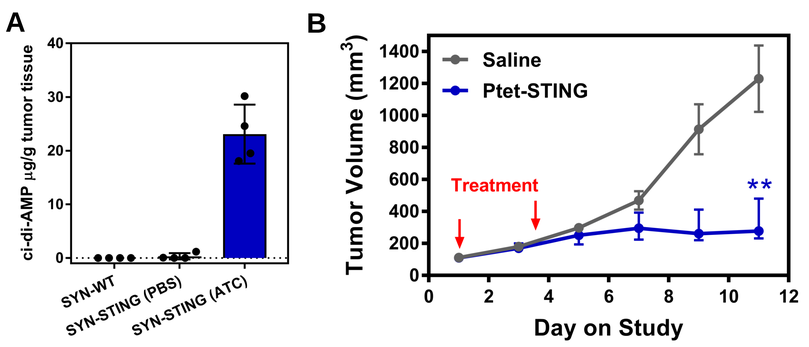


Figure 7: *In Vivo* production of ci-di-AMP and impact on A20 tumor growth and rejection. (**A**) B16F10 tumors were injected with wildtype or ci-di-AMP producing bacteria (1e7 CFUs) via I.T. injection. Four hours post bacterial administration mice were injected with ATC I.P. to induce ci-di-AMP production or PBS. 24 hours post administration tumors were harvested and intratumor ci-di-AMP was measured by LCMS. (**B**) A20 (~40-80mm^3) received 2x doses of saline or bacteria (1e8 CFUs) via I.T. injection. Four hours post bacterial administration mice were injected with ATC I.P. Mean tumor volumes for each experimental group are shown. Significance determined using unpaired t test for tumor volume at the final timepoint. Indicated group compared to Saline; ** P < 0.005.