Bioengineering Bacterially Derived Immunomodulants: A Therapeutic Approach to Inflammatory Bowel Disease

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Supplementary Materials and Methods

Materials

Anti-LAMP1 was purchased from BD Biosciences (San Jose, California). Hoechst 33342 was purchased from AnaSpec Inc. (Fremont, California). 5% washed turkey red blood cells were purchased from Lampire Biological Laboratories (Pipersville, PA).

Immunofluorescence and Colocalization

10,000 J774 macrophages were plated in an 8 well glass chamber overnight. eGFP/mAvrA/AvrA nanoparticles/soluble were given to cells for a period of 6 hours (final eGFP concentration = 150 µg/mL, final mAvrA/AvrA concentration = 12.5 µg/mL). Cells were then washed twice with ice cold PBS and fixed with 3.7% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized with 1% Triton x-100/PBS for 15 minutes at room temperature and blocked with 1% BSA/PBS for 1 hour at room temperature. Lysosomes were labeled with a primary rat anti-LAMP1 antibody (5ug/mL) overnight at 4^{o}C diluted in blocking buffer. Cells were then incubated with a secondary TRITC goat anti-rat antibody (4ug/mL) diluted in blocking buffer for 1 hour at room temperature. Nuclei were stained using Hoechst 33342 (0.2 µM) for 15 minutes at room temperature. Cells with were washed 3 times with PBS (10 minutes per wash) between each step in the staining procedure. 50/50 PBS/glycerol solution was used a mounting media and samples were sealed with a coverslip and nail polish. Images were taken on Zeiss LSM 700 confocal microscope.

MTT Assay

10,000 J774A.1 macrophages were plated in triplicate in each well of a 96 well plate overnight. Nanoparticles (300 µg/mL eGFP, 25 µg/mL AvrA) were incubated with cells for 3 hours. After nanoparticle incubation, media was replaced with 100 µl of fresh media and 10 µl of MTT solution (Biotium). Cells were incubated with MTT reagent for 4 hours. 200 µl of DMSO was then added to each well and wells were mixed vigorously to ensure cells were lysed. Absorbance was measured using a Biotek Synergy 2 Microplate reader at 570 nm and background

absorbance was measured at 630 nm. Background absorbance was subtracted and cytotoxicity was reported as a percentage compared to the negative control (cells with no treatment).

LDH Assay

10,000 J774A.1 macrophages were plated in triplicate in each well of a 96 well plate. Nanoparticles (300 μg/mL eGFP, 25 μg/mL AvrA) were incubated with cells for 6 hrs or 24 hrs. Positive control for maximum (lysed) LDH activity were also run according to the manufacturer's instructions (Pierce LDH Cytotoxicity Assay, Thermo Scientific). 50 μL of supernatant from each sample, negative control, and positive control was transferred to a 96 well plate and 50 μL of reaction mixture was added to each well and mixed. The reaction was protected from light for 30 min and 50 μL of stop solution was added to each well. Absorbance values were measured at 490 and 680 nm using Biotek Synergy2 Microplate reader.

Hemolysis Measurement

Washed red blood cells (RBCs) were diluted to a final concentration of 0.5% v/v in PBS solutions at pH 7.4, 6.5, and 5.4. Nanoparticles/soluble eGFP/mAvrA/AvrA were prepared at a volume of 100 μL at pH 7.4, 6.5, and 5.4 and incubated with an equal volume of the various RBC solutions at 37^{o}C for 1 hour (final eGFP concentration = 150 μg/mL, final mAvrA/AvrA concentration = 12.5 μg/mL). After incubation, samples were centrifuged at 500 g to remove intact RBCs and the hemoglobin that was released into the supernatant was measured in a 96 well plate at an absorbance of 541nm. Relative hemolytic activity was calculated by subtracting the background absorbance of the various RBC solutions from the sample and normalizing to the hemolytic activity of 1% v/v Triton X-100.

Supplementary Figures

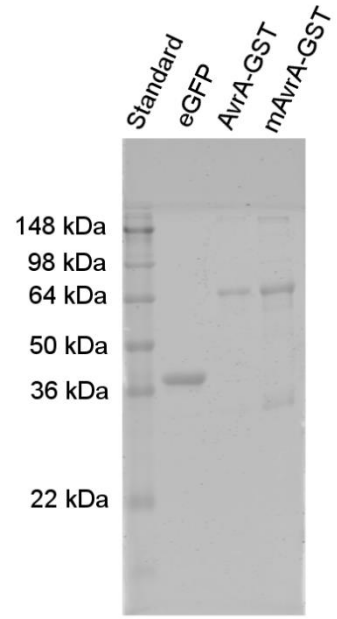

Figure S1. Purity of Ni-NTA purified recombinant eGFP and AvrA fusion proteins. Representative SDS-PAGE gel: lane 1 protein standard, lane 2 eGFP, lane 3 AvrA-GST, and lane 4 mAvrA-GST. In all cases a prominent band is observed at the expected molecular weight.

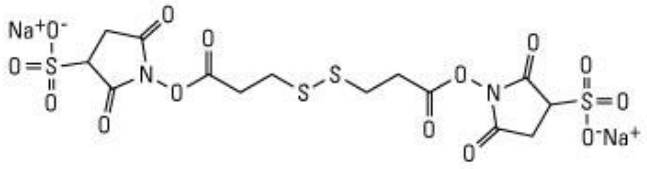

Figure S2. Chemical structure of 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP, Thermo Scientific Pierce). DTSSP is a water-soluble, homo-bifunctional cross-linker that contains a central disulfide bond. DTSSP has two amine-reactive N-hydroxysulfosuccinimide esters at each side of a 12 Å spacer arm.

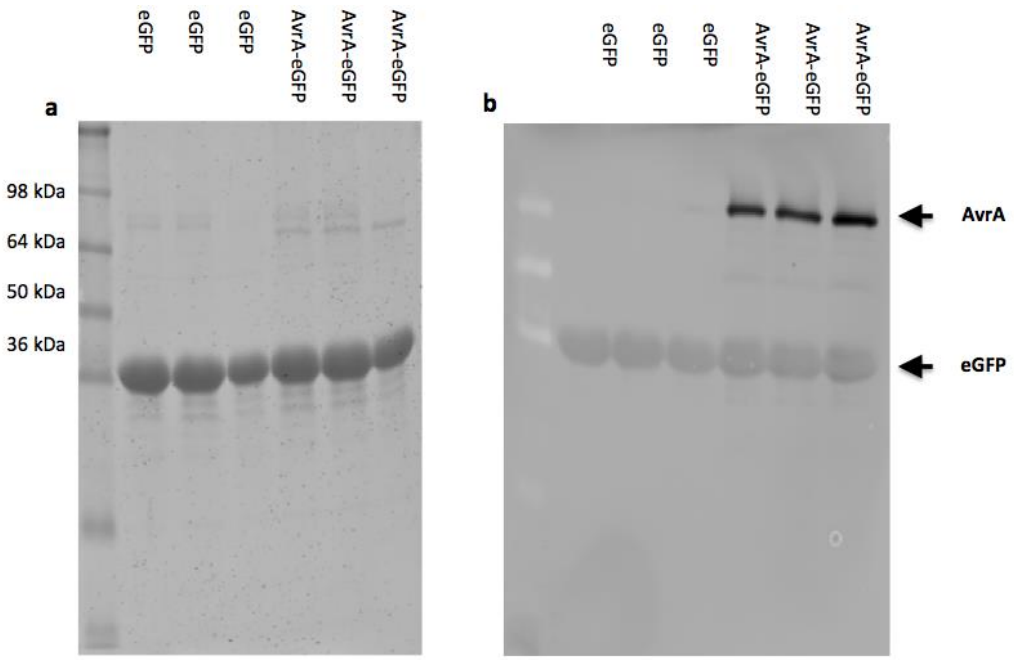

Figure S3. Composition of AvrA-eGFP nanoparticles. (a) Representative SDS-PAGE gel for eGFP and AvrA-GST nanoparticles, and (b) representative western blot for eGFP nanoparticles and AvrA-GST nanoparticles, immunostained with anti-AvrA antibodies and showing native fluorescence of eGFP.

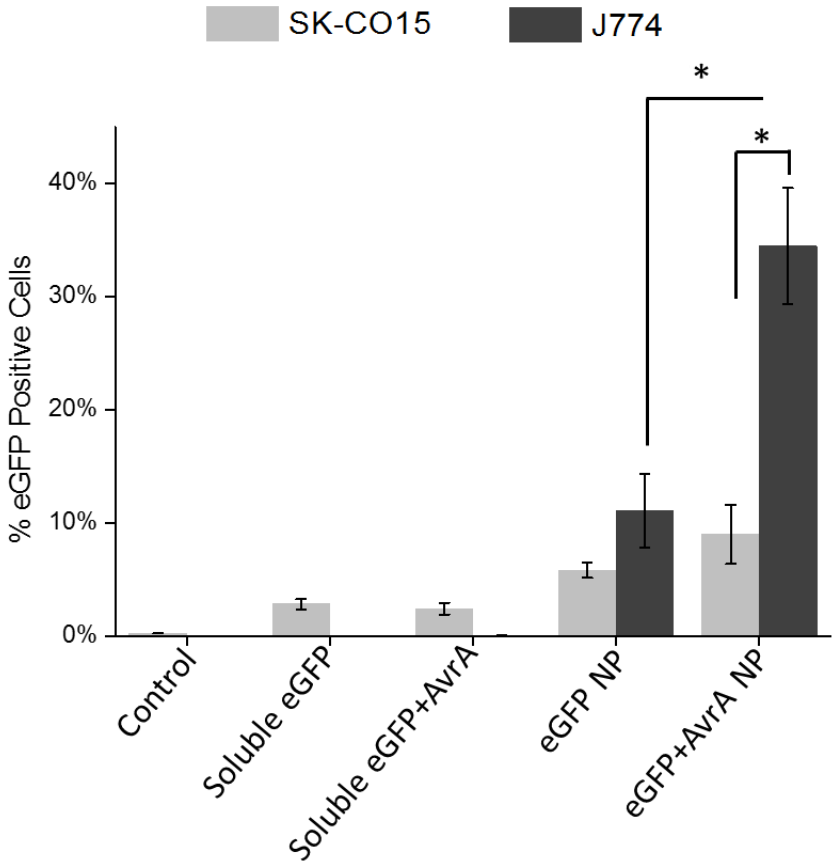

Figure S4. Uptake of AvrA-eGFP protein and nanoparticles. Flow cytometry quantification of soluble eGFP, AvrA and eGFP, or nanoparticle eGFP or AvrA-eGFP uptake in SK-CO15 cells (light gray) and J774A.1 cells (dark gray). (* $p<0.05$, ** $p<0.01$)

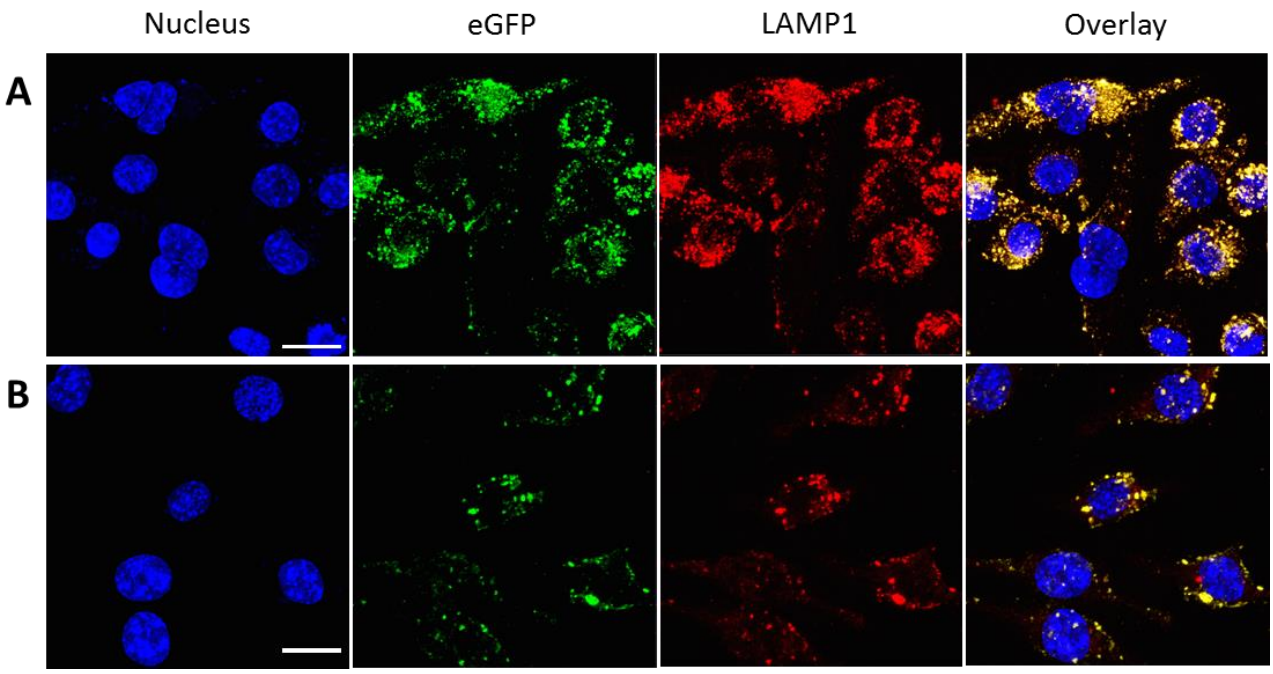

Figure S5. (A) eGFP nanoparticles (green) and (B) eGFP+AvrA nanoparticles were incubated with J774A.1 macrophages for 6 hours and labeled with anti-LAMP1 (red) lysosomal marker. Nuclei were labeled with Hoechst (blue). Overlay images show nanoparticle colocalization with lysosomes (yellow). Images are representative 2D maximum projection images. Scale bars 20 µm

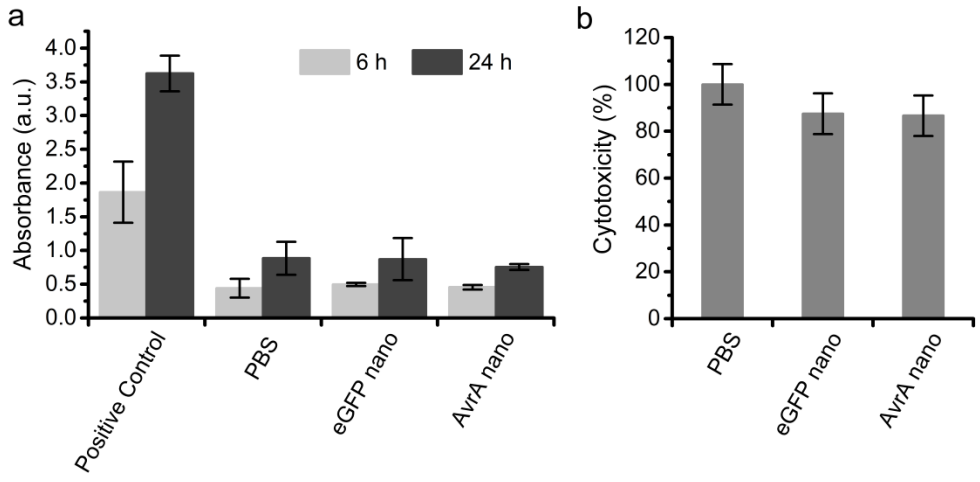

Figure S6. Cytotoxicity of AvrA-eGFP nanoparticles. (a) Lactate dehydrogenase (LDH) activity of J774.A1 cells incubated with nanoparticle formulations of eGFP or AvrA-eGFP for 6 or 24

hours. (b) Cell viability determined by methyl thiazole tetrazolium (MTT) assay for J774.A1 cells treated with soluble or nanoparticle formulations of eGFP or AvrA-eGFP for 3 hours.

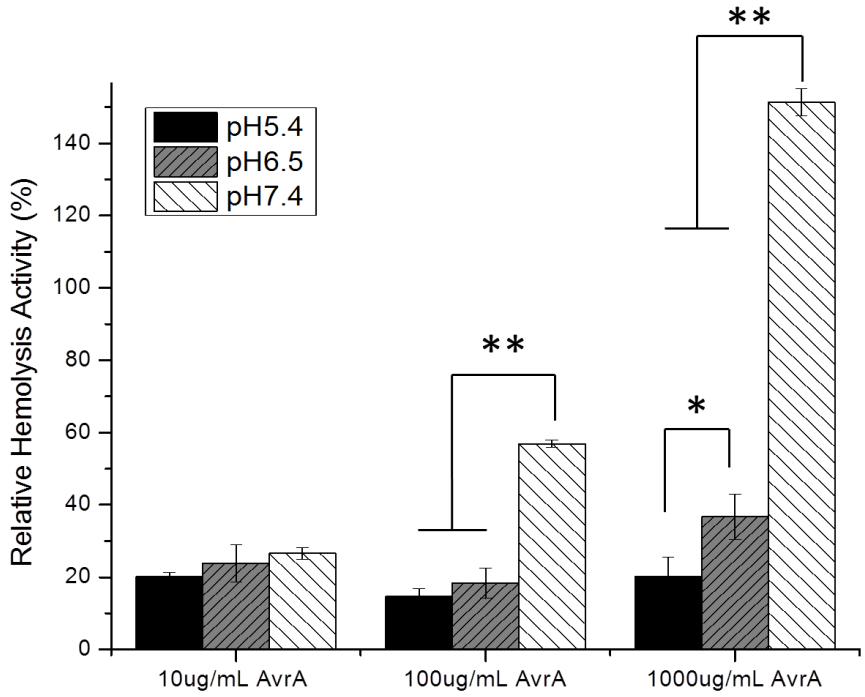

Figure S7. Hemolysis of soluble AvrA-GST at different concentrations and pH values. Relative hemolytic activity is normalized to a positive lytic control, 1% v/v Triton X-100, after subtracting background absorbance at 561nm. (* $p<0.05$, ** $p<0.005$)

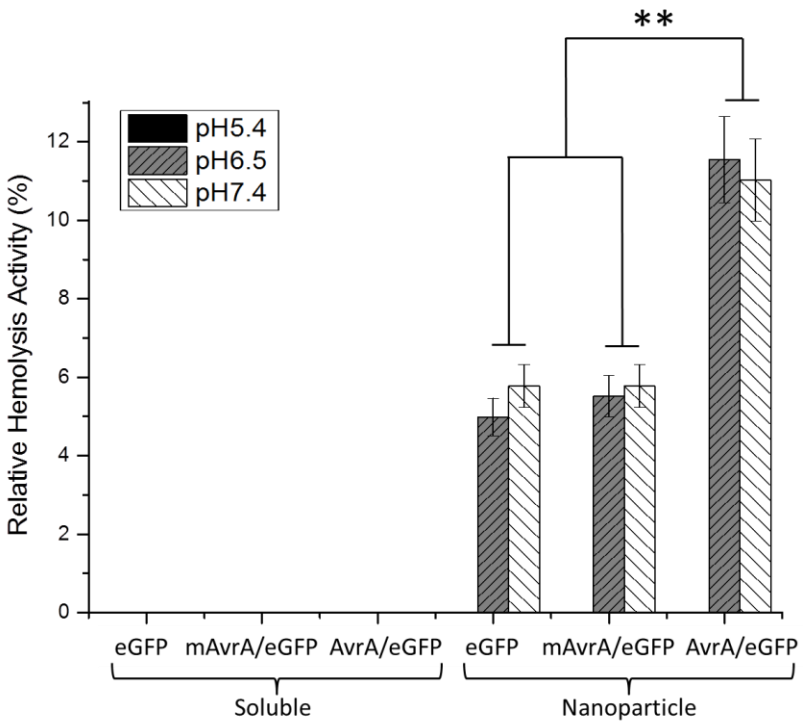

Figure S8. Hemolysis of eGFP (150ug/mL) with or without AvrA or mAvrA (12.5ug/mL) as a function of pH. All soluble samples, as well as pH 5.4 nanoparticle samples, exhibited hemolysis below background. Relative hemolytic activity is normalized to a positive lysis control, 1% v/v Triton X-100, after subtracting background absorbance at 561nm. (** $p<0.005$)

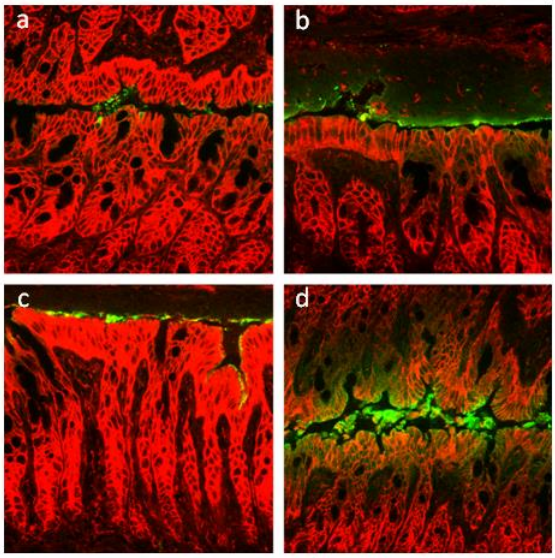

Figure S9. Time course of nanoparticles uptake *in vivo*. Particles were instilled transrectally and imaged at (a) 0.5 hour, (b) 1 hour, (c) 2 hours, (d) 3 hours. Particle uptake is marked by anti-eGFP fluorescence (green). Epithelial cells are counterstained with beta-catenin (red) (40x magnification).

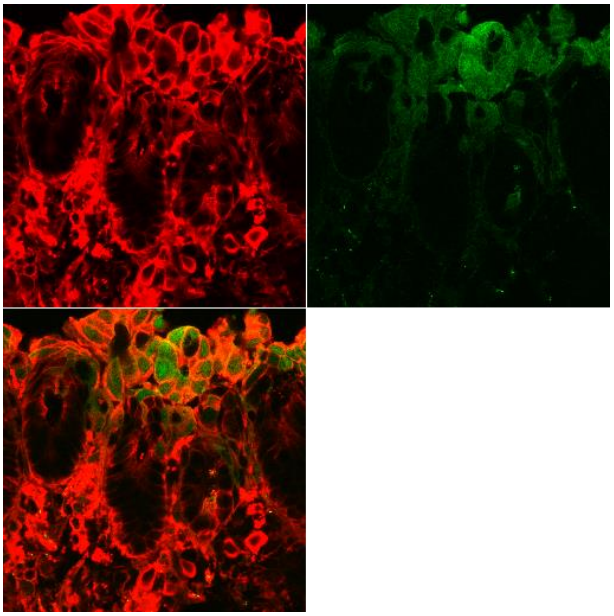

Figure S10. Uptake of AvrA-eGFP nanoparticles in inflamed gut. Particles were instilled transrectally to DSS treated mice (treated for 7 days). Tissue was excised, stained with anti-β-catenin and imaged 4 hours after instillation. Particle uptake is marked by eGFP fluorescence.