Title

Durable, Microbiome-Sparing Phage Suppression of the Mucosa-Adherent Adherent-Invasive *Escherichia coli* (AIEC) Reservoir in Crohn's Disease (the PHAGE-CD program)

Alternate titles considered (title-iteration move kept visible):

- *From fecal to mucosal: testing whether a lytic phage cocktail durably clears the AIEC reservoir that antibiotics cannot*
- *A precision antibacterial for Crohn's: durability, mucosal pharmacodynamics, and resistance pre-emption for AIEC-targeted phage therapy*
- *PHAGE-CD: converting a safety-stage AIEC phage cocktail into mechanism-grade, durably-acting therapy*

Project Summary / Abstract

Crohn's disease (CD) is a chronic, relapsing inflammatory bowel disease in which durable remission remains elusive and current immunosuppressive and biologic therapies target the host immune response rather than any microbial trigger. Adherent-invasive *Escherichia coli* (AIEC), typified by the reference strain LF82, are abnormally enriched on the ileal mucosa of CD patients; they adhere to and invade the intestinal epithelium, survive within macrophages, and promote inflammation. Broad-spectrum antibiotics fail to durably clear mucosa-associated AIEC and collaterally deplete the protective commensal microbiota whose loss is itself associated with CD. Strictly lytic bacteriophages offer a fundamentally different strategy: they kill only their target *E. coli*, spare surrounding commensals, and self-amplify where their host is present.

The AIEC-targeted phage concept is supported by two convergent studies. A lytic coliphage cocktail reduced AIEC burden and reduced DSS-induced colitis in mice after a single dose, and lysed LF82 in ileal biopsies from CD patients (Galtier 2017). A strictly lytic, AIEC-targeted cocktail (EcoActive) was active in vitro against the large majority of a clinical AIEC panel while lysing none of 43 non-*E. coli* commensal strains; a single dose was insufficient, but twice-daily dosing over 15 days protected mice from colitis, and long-term administration to healthy mice did not induce dysbiosis by metagenomics (Titecat 2022). This program does **not** duplicate any ongoing safety trial. Instead it addresses the translational gaps that determine whether phage therapy can become Crohn's first

microbiome-sparing precision antibacterial: (1) defining lytic coverage and the dynamics and fitness cost of phage resistance across a clinically representative, patient-derived AIEC panel, with pre-specified go/no-go criteria; (2) testing in CEACAM6-transgenic mice whether cocktail therapy **durably** suppresses *mucosa-adherent* (not merely fecal) AIEC while leaving the commensal community intact, benchmarked head-to-head against an antibiotic comparator; and (3) building and analytically validating the mucosal pharmacodynamic and resistance-monitoring assays needed to interpret human response, under appropriate FDA and IRB oversight. Together these aims convert a promising safety-stage candidate into a mechanism-anchored, durably-acting therapeutic strategy, with direct extensibility to other AIEC- and pathobiont-associated gut diseases central to NIDDK's mission.

Specific Aims *(one page)*

The problem. CD is associated with mucosal expansion of AIEC (reference strain LF82), which adhere to and invade the ileal epithelium and drive inflammation. Antibiotics neither durably clear the mucosa-associated AIEC reservoir nor spare the protective microbiota; they select for resistance and deepen the dysbiosis associated with CD. No therapy in CD selectively removes AIEC while preserving the commensal community.

The opportunity. Two studies converge on an actionable intervention. A lytic phage cocktail reduced AIEC and reduced DSS-colitis in mice (single dose) and lysed LF82 in CD ileal biopsies (Galtier 2017). A strictly lytic AIEC-targeted cocktail (EcoActive) was active in vitro against the large majority of a clinical AIEC panel while lysing none of 43 non-*E. coli* commensals; twice-daily dosing for 15 days protected mice from colitis, and long-term dosing did not induce dysbiosis (Titecat 2022). The decisive, unanswered questions are whether a lytic cocktail can **durably** suppress the **mucosa-adherent** AIEC reservoir and whether it genuinely spares the host microbiome relative to antibiotics.

Central hypothesis. A strictly lytic, AIEC-targeted phage cocktail, dosed on a regimen anchored to the colitis-protective schedule, will durably suppress mucosa-adherent AIEC and epithelial invasion while preserving commensal diversity — properties that distinguish phages from antibiotics — and these effects can be measured with transferable human-ready assays.

> **Aim 1 — Define lytic breadth, resistance dynamics, and fitness cost of the AIEC-targeted cocktail across patient-derived AIEC.** Assemble an AIEC panel (LF82 plus clinical isolates) and quantify per-phage and whole-cocktail killing, host range, redundancy, and the frequency, kinetics, and fitness cost of resistant escape mutants under single-phage versus full-cocktail pressure. **Outcome / go-no-go:** a quantitative coverage map, an estimated resistance ceiling,

> and a decision rule (pre-specified coverage and escape-suppression thresholds) gating which isolates advance to Aims 2–3.
>
> **Aim 2 — Test durable suppression of mucosa-adherent AIEC with microbiome sparing in CEACAM6-transgenic mice, benchmarked against antibiotics.** In AIEC-colonized CEACAM6-transgenic mice, dose the lytic cocktail versus vehicle versus an antibiotic comparator on a twice-daily regimen anchored to the colitis-protective schedule (Titecat 2022), with pre-specified post-dosing durability/relapse follow-up. **Primary endpoint:** mucosa-adherent (tissue-associated, post-washout) AIEC burden; with epithelial invasion, histologic inflammation, 16S/shotgun microbiome of luminal and mucosal compartments, and longitudinal in vivo phage titers and resistance. **Outcome:** decision-grade evidence on durability and microbiome-sparing versus antibiotic-driven dysbiosis.
>
> **Aim 3 — Develop and analytically validate human-ready mucosal pharmacodynamic and resistance-monitoring assays.** With collaborating IBD sites and under FDA/IRB oversight, validate biopsy- and stool-based assays — mucosa-adherent AIEC quantification, ex vivo phage lysis on biopsy-derived AIEC (extending Galtier 2017), administered-phage recovery/titering, and resistance genotyping/phenotyping (leveraging Aim 1) — with defined input requirements, reproducibility, and acceptance criteria. **Outcome:** a transferable assay package to interpret human mucosal pharmacodynamics, attachable to expanded-access use or future efficacy trials.

Payoff. If a lytic phage cocktail durably suppresses mucosa-adherent AIEC without harming commensals, CD could gain its first microbiome-sparing precision antibacterial, accompanied by a validated, transferable assay toolkit extensible across pathobiont-driven gut diseases.

Significance

Burden and unmet need. CD imposes a lifelong burden of relapsing inflammation, hospitalization, surgery, and disability, and a substantial fraction of patients lose response to immunosuppressive and biologic therapies that target host immunity rather than any microbial trigger. Among candidate microbial drivers, AIEC have among the strongest mechanistic cases: they are enriched on the inflamed ileal mucosa of CD patients, adhere to and invade intestinal epithelial cells, persist within macrophages, and stimulate pro-inflammatory signaling. LF82 is the benchmark strain for this pathotype.

Why current antibacterials are the wrong tool. Broad-spectrum antibiotics do not durably eradicate mucosa-associated AIEC, exert strong selection for resistance, and indiscriminately deplete

commensals whose loss is itself associated with CD. A therapy that selectively removes AIEC while leaving the rest of the community intact would be conceptually distinct from everything currently used in CD.

Why lytic phages, and why now. Strictly lytic bacteriophages are (i) **target-restricted** — able to remove AIEC while sparing commensals; (ii) **self-amplifying** on target, so effective dose tracks pathogen load; and (iii) **non-antibiotic**, avoiding cross-resistance with the antibiotics CD patients already receive. The concept is supported by convergent evidence: a lytic cocktail reduced AIEC and DSS-colitis in mice and lysed LF82 in CD ileal biopsies (Galtier 2017); and a strictly lytic AIEC-targeted cocktail covered the large majority of a clinical AIEC panel in vitro while lysing none of 43 non-*E. coli* commensals, protected mice from colitis on a twice-daily 15-day regimen, and did not induce dysbiosis on long-term dosing (Titecat 2022).

The gap this program closes. Despite this momentum, the field lacks rigorous answers to two questions that are rate-limiting for any therapeutic or regulatory path: does a lytic cocktail **durably** clear the **mucosa-adherent** AIEC reservoir (where invasion and inflammation originate), and does it genuinely spare the **human** microbiome relative to antibiotics? Prior efficacy readouts have emphasized fecal AIEC and colitis prevention rather than durable mucosal clearance, and human mucosal pharmacodynamics for phage therapy are not yet measurable with validated assays. This proposal targets precisely those gaps, which align directly with NIDDK's interest in microbiome-based mechanisms and therapeutics for IBD.

What changes if we succeed. A positive program reframes AIEC management in CD from broad microbial suppression toward **targeted ecological correction**, offers gastroenterologists a tool that does not add to antibiotic exposure, and yields a generalizable "cover the pathobiont → spare the commensals → measure the mucosa" paradigm extensible to other AIEC- and pathobiont-associated diseases.

Innovation

- **A microbiome-sparing precision antibacterial for CD — a category that does not yet exist in IBD.** The program exploits the self-amplifying, host-restricted biology of lytic phages rather than host immunosuppression.
- **Shifting the readout from the fecal compartment to the mucosa-adherent and intra-epithelial reservoir** that is mechanistically responsible for invasion and inflammation, rather than relying on fecal surrogates.
- **Resistance as a primary, quantified endpoint, not an afterthought.** Escape-mutant

frequency, kinetics, and fitness/virulence cost are measured across patient-derived isolates so durability can be predicted rather than assumed, with pre-specified go/no-go thresholds.

- **A head-to-head antibiotic benchmark for collateral damage** built into the in vivo design, converting "microbiome-sparing" from a claim into a measured contrast.
- **A human-ready translational assay toolkit** (biopsy ex vivo lysis, mucosal AIEC quantification, phage recovery, resistance genotyping) that allows mucosal pharmacodynamics to be interpreted in patients under existing regulatory routes — turning a binary safety-trial outcome into mechanism-grade, decision-grade data.

Approach

Overview & experimental logic (the de-risking staircase)

The program advances along a de-risking staircase: **(Aim 1)** establish, in vitro and with pre-specified criteria, that the cocktail covers patient-derived AIEC and suppresses escape; **(Aim 2)** test in the most AIEC-relevant mouse model whether suppression is **durable** and **mucosal** and whether it spares commensals relative to antibiotics; **(Aim 3)** build and validate the human mucosal assays that will be required to read out response in patients. Resistance characterization is the connective tissue: Aim 1 defines it in vitro, Aim 2 tracks it in vivo, Aim 3 makes it measurable in humans.

Aim 1 — Lytic breadth, resistance dynamics, and fitness cost across patient-derived AIEC

Rationale. Durable suppression requires that the cocktail cover the diversity of AIEC encountered clinically and that resistance not rapidly outrun killing. Prior work establishes activity against LF82 and broad in vitro coverage of a clinical AIEC panel with commensal sparing (Galtier 2017; Titecat 2022), but the resistance dynamics and fitness costs that determine durability across a representative panel are not defined.

Design.

1. **AIEC panel.** Anchor on LF82 and expand with clinical AIEC isolates obtained through collaborating IBD programs; confirm AIEC phenotype (adhesion/invasion) and characterize by whole-genome sequencing.
2. **Killing & coverage.** For each isolate, determine per-phage susceptibility and whole-cocktail killing by efficiency-of-plaquing (EOP), planktonic time-kill, and growth-inhibition assays

across a range of multiplicities of infection (MOI). Cross-tabulate phage × isolate to quantify redundancy within the cocktail.

3. **Resistance.** Quantify spontaneous resistant-mutant frequency; compare regrowth kinetics under single-phage versus full-cocktail pressure; sequence resistance loci where feasible.
4. **Fitness cost.** Measure growth rate and, where feasible, adhesion/invasion phenotypes of resistant isolates to test whether escape carries a virulence-relevant cost.

Pre-specified go/no-go criteria (advance to Aims 2–3): [ILLUSTRATIVE]

Criterion	Threshold [ILLUSTRATIVE]
Whole-cocktail coverage of panel	Lyses a pre-specified majority of clinical AIEC isolates by EOP ≥ 0.1
Escape suppression	Full-cocktail pressure delays/abrogates regrowth relative to single phages at target MOI over the assay window
Commensal sparing (confirmatory)	No lysis of a representative non-*E. coli* commensal control set
Isolate prioritization	Broadly covered isolates carried forward; coverage gaps mapped to specific components and reported transparently

Expected outcomes. A quantitative coverage map across patient-derived AIEC, an estimated resistance ceiling, and evidence on whether resistance carries a virulence-relevant fitness cost. We anticipate broad but incomplete coverage and that full-cocktail pressure substantially delays escape relative to single phages.

Potential pitfalls & alternatives.

- *Some clinical AIEC poorly covered.* → Map gaps to specific components to inform future formulation; prioritize broadly covered isolates for Aims 2–3; report gaps transparently rather than concealing them.
- *In vitro resistance overstates in vivo behavior.* → Aim 2 provides the in vivo check; resistance phenotypes are re-evaluated against in vivo escape.

Aim 2 — Durable, mucosal suppression with microbiome sparing in CEACAM6-transgenic mice

Rationale. The decisive clinical questions — whether suppression is **durable** and acts at the **mucosal** surface, and whether commensals are spared relative to antibiotics — remain open. We use **CEACAM6-transgenic mice** as our model because human CEACAM6 confers the AIEC epithelial-adhesion phenotype that is absent in conventional mice; this is our deliberate model choice rather than a feature of the prior cited colitis studies (which used DSS-colitis and AIEC-challenge mouse models; Galtier 2017; Titecat 2022).

Design.

- **Arms:** (1) vehicle; (2) **lytic cocktail**; (3) **antibiotic comparator** (benchmark for collateral microbiome damage). Randomized, blinded scoring.
- **Dosing:** enteral, twice-daily, anchored to the colitis-protective schedule (Titecat 2022), with **pre-specified post-dosing durability/relapse follow-up**.
- **Primary endpoint: mucosa-adherent** AIEC burden (tissue-associated quantification after luminal washout), with **fecal** AIEC for cross-compartment comparison and **epithelial invasion** measures.
- **Inflammation:** blinded histopathology and inflammatory markers.
- **Microbiome sparing:** 16S rRNA and shotgun metagenomics of **luminal and mucosal** samples, comparing cocktail versus vehicle versus antibiotic.
- **In vivo phage dynamics & resistance:** longitudinal phage titers and emergence of resistant AIEC, interpreted against Aim 1.
- **Rigor:** group sizes set by power analysis on the mucosal-AIEC endpoint (biostatistician-determined, 80% power, two-sided α 0.05); randomization, blinded histologic scoring, both sexes included and analyzed as a biological variable; ARRIVE-compliant reporting; authenticated AIEC stocks and sequence-verified phage preparations.

Expected outcomes. Reduction of mucosa-adherent AIEC and inflammation with preserved overall microbiome composition, in contrast to antibiotic-driven dysbiosis. The durability arm reveals whether suppression persists or whether re-colonization/resistance drives relapse — either result is decision-grade.

Potential pitfalls & alternatives.

- *Phages suppress fecal but not mucosa-adherent AIEC, or suppression is transient.* → Test re-dosing schedules; determine, using Aim 1, whether in vivo escape mutants explain relapse.
- *Mucosal delivery limits efficacy.* → Evaluate formulation/dosing-frequency adjustments within the model.

- *Antibiotic arm confounds inflammation read.* → Pre-specify antibiotic, dose, and duration; interpret microbiome contrast independently of the inflammation endpoint.

Aim 3 — Human-ready mucosal pharmacodynamic and resistance-monitoring assays

Rationale. Interpreting whether phage therapy works in patients requires validated mucosal readouts. Ex vivo lysis of LF82 in CD ileal biopsies is feasible (Galtier 2017), providing a foundation for a human pharmacodynamic toolkit aligned with NIDDK's translational priorities. This aim builds the **measurement infrastructure** that safety-stage trials do not, by themselves, provide.

Design. Working with active IBD clinical sites and under appropriate **FDA and IRB oversight**, develop and analytically validate, on patient-derived stool and mucosal biopsies: (i) quantification of mucosa-adherent AIEC; (ii) ex vivo phage lysis on biopsy-derived AIEC; (iii) recovery and titering of administered phages from stool and mucosa; and (iv) genotypic/phenotypic resistance monitoring leveraging Aim 1 methods. Define sample handling, assay reproducibility, input requirements, and acceptance criteria so the toolkit supports pharmacodynamic interpretation. Where investigational phage is administered to individual patients, that use proceeds under the appropriate FDA mechanism for investigational product (single-patient/expanded-access IND as applicable) with IRB oversight, informed consent, and a safety monitoring plan; analysis of residual/biobanked clinical specimens is likewise IRB-governed. **No new efficacy trial is proposed.**

Pre-specified assay acceptance targets (analytical validation): [ILLUSTRATIVE]

Assay	Acceptance target [ILLUSTRATIVE]
Mucosa-adherent AIEC quantification	Defined limit of detection and intra/inter-assay CV below pre-set bounds on biopsy-scale input
Ex vivo biopsy lysis	Reproducible lysis signal on banked AIEC; defined positive/negative controls
Phage recovery/titering	Quantitative recovery within pre-set bounds from spiked stool/mucosal matrices
Resistance monitoring	Concordance between genotypic and phenotypic resistance calls above pre-set threshold

Expected outcomes. A validated, transferable assay package enabling mucosal pharmacodynamic and resistance monitoring in human phage therapy, ready to attach to expanded-access use or future efficacy trials.

Potential pitfalls & alternatives.

- *Biopsy material is limited and variable.* → Prioritize lowest-input assays; validate on banked isolates and consented residual specimens where fresh tissue is constrained.
- *Regulatory/IRB timelines are slow.* → Assay development on existing isolates and residual samples proceeds in parallel so progress does not depend on enrollment.

Timeline *(illustrative, 5-year R01-scale)* [ILLUSTRATIVE]

- **Year 1 [ILLUSTRATIVE]:** Aim 1 panel assembly and lytic/resistance/fitness characterization; initiate IRB groundwork and assay design for Aim 3.
- **Years 2–3 [ILLUSTRATIVE]:** Aim 2 in vivo dosing, durability, and microbiome studies with antibiotic benchmark; complete Aim 1; begin Aim 3 analytical validation on banked isolates.
- **Years 4–5 [ILLUSTRATIVE]:** Aim 2 durability/relapse analyses; Aim 3 validation on patient mucosal/stool specimens under FDA/IRB oversight; integration and dissemination.

Budget Justification *(modular R01-style sketch — illustrative, not a quote)* [ILLUSTRATIVE]

This is a modular R01 request of **[ILLUSTRATIVE]** direct costs of **$250,000/year [ILLUSTRATIVE]** for **5 years [ILLUSTRATIVE]**.

- **Personnel [ILLUSTRATIVE]:** PD/PI (microbiology/IBD; ~2.4 calendar months); co-investigators in phage biology, gastroenterology, and bioinformatics (effort each **[ILLUSTRATIVE]**); one postdoctoral scientist and one research technician (Aims 1–2); part-time study coordinator/regulatory specialist (Aim 3); biostatistician effort for Aim 2 power and Aim 3 validation.
- **Supplies/consumables [ILLUSTRATIVE]:** bacterial/phage culture, EOP/time-kill reagents, resistance assays, sequencing library preparation.
- **Animal costs [ILLUSTRATIVE]:** CEACAM6-transgenic mouse husbandry, per-diems, and

procedures for Aim 2.

- **Sequencing/core services [ILLUSTRATIVE]:** 16S and shotgun metagenomics; histopathology core.
- **Clinical/regulatory [ILLUSTRATIVE]:** specimen collection, processing, and biobanking at collaborating sites; IRB submission and maintenance; regulatory submissions associated with investigational-product use (Aim 3).
- **Other [ILLUSTRATIVE]:** publication, travel, and data-sharing costs.

Final-year budgets assume no escalation beyond standard institutional rates **[ILLUSTRATIVE]**. Investigational phage material is supplied by the manufacturing collaborator under appropriate agreements; the budget does **not** assume in-house phage manufacturing.

Vertebrate Animals (Aim 2)

- **Species/justification.** CEACAM6-transgenic mice are required to recapitulate human CEACAM6-mediated AIEC epithelial adhesion, which conventional mice do not support; no non-animal system reproduces durable mucosal colonization with host-relevant invasion.
- **Procedures.** AIEC colonization; oral phage, antibiotic, or vehicle dosing; serial and terminal sample collection; humane euthanasia for mucosal and tissue analyses.
- **Numbers.** Group sizes (**[ILLUSTRATIVE]**) are the minimum required for statistical power on the mucosa-adherent AIEC endpoint (biostatistician-determined), with randomization, blinded histologic scoring, and inclusion of both sexes.
- **Welfare / 3Rs.** IACUC-approved protocol with veterinary oversight; analgesia and pre-specified humane endpoints minimize distress. *Replace* — in vitro coverage/resistance assays (Aim 1) maximally filter candidates before any animal work. *Reduce* — pre-specified power and shared controls. *Refine* — validated humane endpoints; ARRIVE-compliant reporting.

Human Subjects / Clinical Specimens (Aim 3)

This project does **not** initiate a new interventional efficacy trial. Aim 3 involves human-derived stool and mucosal biopsy specimens and assay development to support pharmacodynamic and resistance monitoring of investigational phage therapy.

- **Oversight & protections.** Any administration of investigational phage to individual patients proceeds under the appropriate FDA mechanism for investigational product

(single-patient/expanded-access IND as applicable), with **IRB oversight**, informed consent, and a data and safety monitoring plan at participating sites. Collection and analysis of residual/biobanked clinical specimens are IRB-governed.

- **Contextual trials.** Contemporaneous safety-stage phage trials in CD are referenced as context only, not as deliverables of this award; this aim builds measurement infrastructure those trials do not by themselves provide.
- **Inclusion.** Sex/gender, age, and minority inclusion follow NIH policy; assays are validated across the demographic range of enrolled CD patients, and sex is analyzed as a biological variable where specimen numbers permit.

Team & Environment *(template — fill with real names)*

- **Contact PD/PI — [Name, Institution] [ILLUSTRATIVE]:** IBD microbiology/translational lead; overall direction, Aims 1–2.
- **Co-Investigator (Phage Biology) — [Name, Institution] [ILLUSTRATIVE]:** lytic phage characterization, host-range matrix, and resistance (Aim 1).
- **Co-Investigator (AIEC Pathobiology) — [Name, Institution] [ILLUSTRATIVE]:** AIEC/CEACAM6 model expertise (Aim 2).
- **Co-Investigator (Clinical Gastroenterology) — [Name, Institution] [ILLUSTRATIVE]:** patient isolates, biopsy access, and FDA/IRB pathway (Aim 3).
- **Co-Investigator (Microbiome Bioinformatics) — [Name, Institution] [ILLUSTRATIVE]:** 16S/shotgun analysis (Aim 2).
- **Biostatistician — [Name, Institution] [ILLUSTRATIVE]:** power analysis (Aim 2) and assay-validation statistics (Aim 3).
- **Industry collaborator [ILLUSTRATIVE]:** provider of the strictly lytic AIEC-targeted cocktail and manufacturing/regulatory input under appropriate agreements (no in-house manufacturing assumed).
- **Environment:** academic medical center with BSL-2 microbiology, a transgenic animal facility, sequencing and histopathology cores, and an IBD clinical program with regulatory/IRB infrastructure. **Alternate/complementary funders [ILLUSTRATIVE]** include **NIAID** (antibacterial/phage therapeutics) and the **Crohn's & Colitis Foundation** (IBD-focused mechanisms).

References

1. Galtier M, et al. Bacteriophages Targeting Adherent-Invasive *Escherichia coli* Strains as a Promising New Treatment for Crohn's Disease. *J Crohns Colitis.* 2017;11(7):840–847. https://doi.org/10.1093/ecco-jcc/jjw224
2. Titecat M, et al. Safety and Efficacy of an AIEC-targeted Bacteriophage Cocktail in a Mice Colitis Model. *J Crohns Colitis.* 2022;16(10):1617–1627. https://doi.org/10.1093/ecco-jcc/jjac064

> Citations restricted to the two allowed references. Trial registry identifiers, specific named laboratories/companies, and the precise phage-count of any specific commercial cocktail were removed from the body because they are not supported by these two references; verify and reinstate against primary sources before submission.