Title:	**Feasibility Study of Intra-Tumoral Lipopolysaccharide Immunotherapy for Intra-Abdominal Tumors (RIOT)**
Drug or Device Name(s):	**Lipopolysaccharide (LPS; E. coli 0113)**
FDA IND	**28677**
Sponsor:	**Investigator-sponsored trial.**
IRB RC Number	**2022-263-AGH**
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ABBREVIATIONS AND DEFINITIONS OF TERMS

AE	Adverse event
AGH	Allegheny General Hospital
AHNCI	Allegheny Health Network Cancer Institute
BMI	Body Mass Index
BSA	Body Surface Area
CFR	Code of Federal Regulations
CTCAE	Common Terminology Criteria for Adverse Events
DSMB	Data Safety Monitoring Board
FDA	Food and Drug Administration
G-CSF	Granulocyte-Colony Stimulating Factor
GMP	Good Manufacturing Practice
HIPAA	Health Insurance Portability and Accountability Act
IL	Interleukin
IND	Investigational New Drug
IRB	Institutional Review Board
LPS	Lipopolysaccharide
MRN	Medical Record Number
NSAID	Non-steroidal Anti-inflammatory Drug
SAE	Serious Adverse Event
SIRS	Systemic Inflammatory Response Syndrome
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
WPH	West Penn Hospital

ABSTRACT

Context: (Background)

Immunotherapy for advanced cancers of the digestive tract can be quite effective, but not all tumors are responsive to this type of treatment. There is intense interest in new methods to convert non-responsive tumors into responsive tumors. One such method is to inject chemical constituents of micro-organisms into tumors in order to stimulate the immune system to recognize the tumor as foreign and mount an immune response to treatment.

Objectives: (primary and important secondary objectives)

The objective of this study is to determine the safety and feasibility of injecting a bacterial cell wall chemical, called lipopolysaccharide (LPS), into intra-abdominal tumors of digestive origin during a routine laparoscopic diagnostic staging procedure in a patient who is expected to undergo a subsequent definitive surgical procedure. The tumor tissue will be biopsied during standard-of-care procedures before and after treatment to examine whether immune recognition is occurring as a result of the injection.

Study Design:

Phase I safety and feasibility trial in 12 patients with advanced metastatic digestive cancer.

Setting/Participants:

We will enroll 12 patients undergoing diagnostic staging laparoscopic surgery at West Penn Hospital or Allegheny General Hospital for advanced metastatic digestive malignancy who are expected to undergo a second, definitive abdominal operation several weeks later following the diagnostic staging procedure.

Study Interventions and Measures:

Participants will undergo a single injection of LPS into an intra-abdominal tumor of digestive tract origin, and a control injection of a second tumor with the carrier solution only.

Potential participants will be undergoing a preliminary diagnostic laparoscopic procedure and biopsy (standard clinical care). Depending upon the findings at the time of laparoscopy, participants who are deemed eligible to undergo a second, definitive, surgical procedure will be enrolled into the study and undergo injection of the study drug into one abdominal tumor and a control injection of the carrier solution without the study drug into a second tumor. Potential participants who are deemed ineligible for subsequent procedures will be considered screen failures.

PROTOCOL SYNOPSIS

Study Title	**Feasibility Study of Intra-Tumoral Lipopolysaccharide Immunotherapy for Intra-Abdominal Tumors**
Funder	Institutional Funds
Clinical Phase	Phase I
Study Rationale	The use of immunotherapy can be quite effective in treating advanced abdominal cancers, but few patients are eligible or responsive to therapy due to immune tolerance of tumor tissue or immune cell exhaustion. There is intense interest in immunomodulatory therapies, which are designed to alter the tumor environment toward a more adaptive and responsive state, which would activate anti-tumor mechanisms by the patient's immune system. One approach has been to inject microbial components directly into tumors, with the intention of inducing recognition of tumor tissue as foreign by the immune system. Intra-tumoral delivery has been applied mostly to skin tumors, since they are accessible for injection, whereas visceral tumors are relatively inaccessible. This protocol describes injection of an immunomodulatory agent directly into intra-abdominal metastatic tumors of digestive tract origin at the time of standard-of-care diagnostic staging laparoscopy in anticipation of a subsequent, more definitive surgical operation. The agent, lipopolysaccharide (LPS) from *E. coli* 0113, has been administered to normal volunteers and cancer patients intravenously and intra-dermally in prior studies of sepsis and as a candidate anti-neoplastic agent.
Study Objective(s)	**Primary** • To determine the feasibility and safety of intra-tumoral laparoscopic injection of LPS into intra-abdominal tumors. **Secondary** • To assess alterations in the tumor immune microenvironment in response to the experimental immunomodulatory agent by examining pre- and post- therapy biopsies taken as part of routine clinical care.
Test Article(s) *(If Applicable)*	The study drug is lipopolysaccharide from *E. coli* 0113, a bacteria cell component used as an immunomodulatory agent.
Study Design	Single-arm, open label, phase I safety and feasibility study, with correlative translational studies.
Subject Population	**Inclusion Criteria** • Adult patients (age 18-99) with biopsy-proven advanced digestive malignancy, including metastatic or recurrent

Key criteria for Inclusion and Exclusion:	tumors, who are scheduled to undergo a preliminary laparoscopic procedure and are found to be eligible for a definitive interval invasive surgical procedure. • Participants must have at least two index intra-abdominal soft tissue tumors that are grossly visible, >1cm^3 in volume separate from visceral and neurovascular structures, and amenable to biopsy and injection with study drug or an equal volume of the carrier solution without drug (control injection). **Exclusion Criteria** • Pregnant patients • Patients with a history of allergy to the investigational agent • Patients on active immunosuppressive therapy including corticosteroids • Patients on active chemotherapy or radiotherapy (must be discontinued 4 weeks prior to study interventions) • Active infection requiring systemic therapy or causing fever (temperature >38.1C) or unexplained fever within 7 days prior to the date of investigational product administration • Patients who are deemed ineligible for a subsequent definitive operation at the time of diagnostic laparoscopy • Adverse events from prior therapy that have not resolved to < grade 1 according to CTCAE version 5 prior to enrollment • Medical contra-indication or allergy to acetaminophen or NSAID
Number Of Subjects	12 patients to be treated on protocol; (additional patients may be enrolled but deemed ineligible at the time of laparoscopy)
Study Duration	Each subject's participation will be limited to a screening visit, an intervention visit (at the time of standard-of-care laparoscopic surgery), a follow-up telephone visit, and a final visit expected to be 14-30 days following the intervention visit (at the time of subsequent definitive surgical procedure). For an individual patient, the study is expected to last approximately one month. The entire study is expected to last up to 18 months.
Study Phases **Screening** **Intervention** **Follow up**	(1) Screening: screening for eligibility and provision of consent documents (2) Intervention/open label treatment: informed consent and injection of the study drug and control solution into intra-abdominal soft tissue tumors at the time of standard-of-care diagnostic laparoscopy

	(3) Follow up: Participants will be interviewed and examined at the time of subsequent definitive surgery 14-30 days following the diagnostic laparoscopy.
Efficacy Evaluations	Not applicable.
Pharmacokinetic Evaluations	Not applicable.
Safety Evaluations	Drug-related toxicity will be evaluated using the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.
Statistical And Analytic Plan	Rates of adverse events and feasibility failures will be calculated using standard descriptive statistical methods.
DATA AND SAFETY MONITORING PLAN	The safety outcomes of this study will be monitored by the AHNCI DSMB.

TABLE 1: SCHEDULE OF STUDY PROCEDURES

Study Phase	**Screening**	**Intervention**	**Telephone Follow-up**	**Study Close**
Visit Number	**1**	**2**	**3**	**4**
Review inclusion/exclusion criteria	X			
Review demographics/medical history/medications/laboratory studies/pregnancy test	X			
Physical examination including vital signs (heart rate, blood pressure, temperature)	X	X		
Informed consent	X	X		
Standard-of-care diagnostic laparoscopy and biopsy		X		
Injection of study drug and control injection		X		
Immediate adverse event assessment		X		
Delayed adverse event assessment			X	X
Second standard-of-care surgery/biopsy and study close				X

Figure 1: Study Diagram

1 BACKGROUND INFORMATION AND RATIONALE

1.1 Introduction

Immunotherapy for metastatic gastrointestinal and other cancers is gaining utility in clinical oncology. However, effective immunotherapy requires recognition of a tumor as a foreign entity by the immune system, to which an adaptive response can be mounted. Because most tumors are able to suppress or escape recognition by the immune system, there is intense interest in developing treatment methods to stimulate immune recognition and destruction of malignant cells. Many of these approaches involve the injection of material from bacteria, viruses or other micro-organisms directly into tumors, with the hope that the foreign material will incite recognition and response to the tumor by the patient's immune system.

In this protocol, we describe the intra-tumoral injection of a bacterial cell well component (lipopolysaccharide, LPS) directly into intra-abdominal metastatic tumors of digestive tract origin in patients with metastatic cancer. This agent has been studied extensively as a model of sepsis, having been administered intravenously to many healthy research volunteers. It has also been injected as a subcutaneous dose to patients with advanced cancer. However, to our knowledge, direct injection into tumors has not been attempted with this agent.

In order to achieve directed intra-tumoral delivery into tumors, we propose to study patients who are undergoing diagnostic laparoscopy as a standard-of-care procedure in anticipation of a subsequent more definitive procedure 14-30 days later. This will allow for a before-and-after assessment of injected tumors to assess the immune response to the study drug. In order to avoid toxicity, only metastatic tumors attached to soft tissues of the abdomen (e.g., abdominal wall, omentum, or diaphragm) will be injected; visceral metastases will not be injected with the investigational agent.

1.2 Name and Description of Investigational Product or Intervention

The investigational drug is lipopolysaccharide (LPS) derived from *E. coli* 0113. LPS is the dominant component of the gram-negative bacterial cell wall and has long been recognized as the principal chemical stimulus for sepsis during infections. In addition to stimulating systemic toxicity, LPS is recognized directly by immune cells as a signal of infection through a cellular receptor known as Toll-like Receptor 4 (TLR4), which belongs to a family of pattern recognition receptors evolved to detect microbial material in human tissue. These receptors, when stimulated, result in activation of immune cells to express transcription factors and cytokines that alter the tumor immune microenvironment toward an adaptive anti-tumor state. The use of natural or synthetic molecules aimed at stimulating TLR4 and other pattern recognition receptors is an extremely active area of immunotherapy research. Intra-tumoral injection of LPS into abdominal tumors is a similar idea to existing treatments for cutaneous melanoma, in which live attenuated micro-organisms are injected into tumors. However, the current protocol uses no living organisms, substituting instead the active cell wall constituent recognized by the human immune system.

1.3 Findings from Non-Clinical and Clinical Studies

1.3.1 Non-Clinical Studies

The mechanism of action of LPS as an anti-neoplastic therapy has been extensively studied and is presumed due to its engagement of toll-like receptors (TLRs) on human immune cells in a manner that stimulates adaptive immune response to tumor antigens, as reviewed by Boushehri et al[1]. Specifically, LPS is recognized by the pattern-recognition receptor TLR4, which subsequently causes secretion of the pro-inflammatory cytokines TNF-α and IL-1β by macrophages and type 1 interferons and IL-12 by dendritic cells[2]. By conditioning the cytokine milieu of the tumor microenvironment, it is proposed that TLR4 activation can promote infiltration by tumor-specific lymphocytes and thus break immune tolerance[1].

Intravenous injection of LPS was studied in syngeneic mouse sarcoma models by Berendt el al., who demonstrated immunogenic tumor regression and long-term durability of response[3]. In this model, a single intravenous injection of 5ug of LPS was utilized. Intradermal and intratumoral LPS injections were also studied in animal models, including a model of glioma-bearing mice. In the latter, repeated weekly injections of 400μg of LPS led to durable tumor regression in 19 out of 20 mice[4]. A related study in a rat model showed not only regression of tumors following LPS injection, but also evidence of immunologic memory upon re-challenge with subsequent tumor injections[5]. Finally, a melanoma-bearing mouse model of a dose of 500ng of LPS administered intra-tumorally resulted in complete elimination of melanoma with evidence of dendritic cell activation in the tumor microenvironment[6].

1.3.2 Clinical Studies

1.3.2.1 Human Pharmacokinetics

There are no existing pharmacokinetic studies in humans related to intra-tumoral LPS injection. There is an extensive experience with intravenous LPS injection described in section 1.3.2.2, although no specific pharmacokinetic information is available in an extensive literature search.

1.3.2.2 Clinical Studies in Adults

Intra-tumoral injection of LPS has not been studied in a clinical trial in humans, to our knowledge. Intra-dermal injection of LPS (isolated from *Pantoea agglomerans*) was administered to ten advanced cancer patients with a variety of tumor types in a dose escalation study by Goto et al[7]. From a starting dose of 0.4ng/kg (based on prior IV dosing studies), the dose was escalated to 600 or 1800ng/kg (per protocol) with minimal side effects in all of the five patients who received the higher dose. Dosing was twice weekly for up to four months in duration. The toxicity seen with this dose was self-limited fever (<12 hours) in 4/5 patients, mild fatigue in 4/5 patients and mild nausea in 3/5 patients. There were no hepatic, renal, hematologic or cardiopulmonary toxicities and no hypotension[7]. Although the authors do not report circulating LPS levels following intra-dermal injection, they did report peaks in the circulating levels of TNF, IL-6 and G-CSF in the hours following injection with a peak concentration at 1-2 hours. Tumor response, either serologic or radiographic, was

observed in three out of four evaluable patients who received the full intended dose; with stable disease in the fourth patient[7].

Intravenous LPS was utilized in a single Phase 1 study as an anti-cancer therapy in 24 patients with advanced cancer[8]. Patients were treated biweekly with 5 ng/kg LPS from *Salmonella abortus equi*. Oral ibuprofen (800mg) was given concomitantly to reduce constitutional toxicities seen in preliminary studies (fever, chills, fatigue, headache, and myalgia). LPS injection induced systemic release of TNF- α, IL-6, IL-8, and G-CSF. Tumor response was assessed at eight weeks, at which time there were four partial responses, nine patients with stable disease, and seven patients with disease progression[8].

Intravenous LPS has been utilized extensively in human studies as a model for sepsis, in numerous studies dating back to the 1960s, as reviewed by van Lier et al[9]. Many of these studies utilized the LPS from *E. coli* 0113, the same strain proposed in the current protocol[10–14], with a typical dose range of 2-4ng/kg. As documented in these references, there are reproducible physiologic and serologic effects peaking within 1-2 hours following intravenous injection, including the systemic release of a number of cytokines and metabolic markers. Calvano et al describe a study of 66 volunteers in normal health, in whom LPS injection reliably recapitulated the systemic inflammatory response syndrome (SIRS)[11].

1.4 Selection of Drugs and Dosages

LPS derived from *E. coli* 0113 is available as a GMP-compliant formulation through List Laboratories, Inc. (Campbell, CA, USA). At least three reports describing the use of the formulation from List Laboratories in intravenous administration are available[15–17].

Taken together, after reviewing the dosing regimens above, a single dose of 1μg of LPS is proposed to be injected into tumor tissue during standard-of-care diagnostic laparoscopy, a dose less than 1% of the intra-dermal dose that was safely administered in the study by Goto et al with only mild and self-limited constitutional toxicities (126μg for a typical 70kg patient). The dose proposed here is smaller, but would be concentrated in the tumor microenvironment and twice the level shown to be efficacious in the preclinical rat melanoma model of Maito et al.

If this protocol is successful in demonstrating feasibility and translational (biomarker) signals of immune activity, then a formal dose-finding study will be performed subsequently to determine the ideal dose of intra-tumoral injection of the investigational agent.

1.5 Relevant Literature and Data

Intra-tumoral immunotherapy, as a concept, predates modern targeted immunotherapy, extending back to anecdotal observations of tumor regression in the setting of infection. The canonical example was the work of Coley, who observed spontaneous regression of sarcoma in the setting of infection, and subsequently utilized a mixture of toxins derived from *Streptococcus pyogenes* and *Serratia marcescens* as a therapeutic adjuvant in the treatment of cancer[18,19]. The active component of Coley's toxin was later demonstrated to be lipopolysaccharide (LPS)- the predominant glycolipid found in the outer membrane of gram-negative bacteria[20].

Early studies focused on the use of LPS as an antineoplastic therapy showed promising effects in intravenous injection in murine models and in humans[3,8]. Using LPS derived from *Salmonella abortus equi*, a maximum tolerated intravenous dose was established to be 10 ng/kg, albeit with significant constitutional toxicity (fever, chills, myalgia, fatigue) requiring high dose NSAID co-administration[8]. A follow-up phase II clinical trial in patients with non-small cell lung cancer and colon cancer demonstrated two partial and one complete responses among eleven patients, with the complete response exceeding 36 months in duration[21].

The mechanism of action of LPS as an anti-neoplastic therapy has been extensively studied and is presumed due to its engagement of toll-like receptors (TLRs) on human immune cells in a manner that stimulates adaptive immune response to tumor antigens, as reviewed by Boushehri et al[1]. Specifically, LPS is recognized by the pattern-recognition receptor TLR4, which subsequently causes secretion of the pro-inflammatory cytokines TNF-α and IL-1β by macrophages and type 1 interferons and IL-12 by dendritic cells[2]. By conditioning the cytokine milieu of the tumor microenvironment, it is proposed that TLR4 activation can promote infiltration by tumor-specific lymphocytes and thus break immune tolerance[1].

Due to the systemic toxicity of intravenous LPS administration, and based on the recognition of the tumor microenvironment as the ultimate target of immunomodulatory therapy, efforts were made to study intra-dermal[7] and intra-tumoral injection of LPS[4–6]. The latter modality showed promising effects in rodent models of subcutaneous implanted glioma as well as melanoma. To our knowledge, intra-tumoral injection of LPS as a TLR4 agonist for immunotherapy has not been studied in human patients, despite its investigational use as an intravenous and intradermal agent in cancer patients and normal volunteers[7,8,21,22].

Although intra-tumoral injection of TLR receptor agonists and other immunotherapeutic agents is an intense area of active research[23], the utility has been largely relegated to cutaneous neoplasms because of the complexities inherent in delivery of repeated intra-tumoral immunotherapy dosing into visceral or organ-space targets. We propose to overcome this limitation by capitalizing on the common clinical scenario in which patients who are potential candidates for a major cancer operation undergo a preliminary laparoscopic procedure.

Patients with metastatic intra-abdominal digestive malignancies often undergo laparoscopy for the purpose of assessing the feasibility of a definitive operation to remove metastatic tumors (cytoreductive surgery). The time interval between the laparoscopy and the definitive operation varies from patient to patient, but is commonly up to four weeks while preparations are made for the definitive operation. We propose to utilize the initial procedure as an opportunity to inject tumors with an experimental immunomodulatory agent or control solution, and to capitalize on the second surgery to examine tumor response 14-30 days later.

Intra-abdominal metastatic tumors can be attached to visceral or parietal peritoneal surfaces, as well as intrinsic (parenchymal) to organs such as the liver or spleen. In this

study, only tumors attached to parietal (soft tissue) surfaces will be injected with the investigational agent, in order to avoid organ toxicity or major neurovascular structures.

1.6 Compliance Statement

This study will be conducted in full accordance with all applicable Allegheny Health Network Research Policies and Procedures and all applicable Federal and state laws and regulations including 45 CFR 46, 21 CFR Parts 50, 54, 56, 312, 314 and 812 and the Good Clinical Practice: Consolidated Guideline approved by the International Conference on Harmonisation. All episodes of noncompliance will be documented.

The investigators will perform the study in accordance with this protocol, will obtain consent, and will report unanticipated problems involving risks to subjects or others in accordance with the Allegheny Health Network IRB Policies and Procedures and all federal requirements. Collection, recording, and reporting of data will be accurate and will ensure the privacy, health, and welfare of research subjects during and after the study.

2 STUDY OBJECTIVES

2.1 Primary Objective (or Aim)

The primary objective of this study is to determine whether LPS (*E. coli* 0113) can be safely and feasibly delivered to intra-abdominal tumors during laparoscopic surgery in a single dose of 1μg.

2.2 Secondary Objectives (or Aim)

The secondary objective is to determine whether biomarkers related to the tumor immune microenvironment are altered following LPS delivery using biopsies obtained during standard-of-care procedures.

3 INVESTIGATIONAL PLAN

3.1 General Schema of Study Design

This is an open-label, Phase I safety and feasibility study.

3.1.1 Screening Phase

Potential participants will be identified from among patients treated by the physician-investigator. Candidates who meet criteria will then be approached by the physician-investigator to assess interest in participation. Interested candidates will be provided with the informed consent form to review.

3.1.2 Intervention at the time of standard-of-care laparoscopy

Upon enrollment into the study, patients will be preparing for standard-of-care surgery. They will undergo informed consent and education regarding the study procedures. On the day of surgery, patients will be cared for according to standard clinical procedures, which includes multi-modal analgesia (acetaminophen and NSAIDs). An 18-gauge core needle biopsy of the intended target lesion during is taken as part of routine clinical care

during surgery. At the conclusion of a standard-of-care surgery and tumor biopsy, two tumors will be selected for intervention. These will be parietal (non-visceral) metastatic tumors from biopsy-proven primary digestive malignancies. Tumors attached to visceral organs will not be eligible for injection, whereas non-visceral tumors attached to the abdominal wall, parietal peritoneum or omentum will be eligible for injection. Tumors adjacent to major neurovascular structures such as mesenteric or other major blood vessels will not be eligible for injection. One tumor will be injected with 1μg LPS (investigational drug) and the second tumor will be injected with an equal volume of carrier solution only (control injection of normal saline), just prior to the conclusion of surgery and emergence from anesthesia. All injections will be performed, over approximately one minute per injection, by the physician investigator, who will also be the attending surgeon for the standard-of-care operations.

Participants will be observed in the operating room and recovery room according to standard clinical protocols. Vital signs will be monitored for heart rate, blood pressure and temperature alterations. Patients emerging from anesthesia have predictable alterations in vital signs including tachycardia, bradycardia, hypotension, hypertension, hypothermia and hyperthermia. Therefore, only sustained alterations in vital signs that persist into the postoperative care unit (recovery room) will be considered to constitute potential adverse events. An adverse event will be defined as treatment-related when it cannot reasonably be attributed to routine, standard-of-care laparoscopy and biopsy. Patients are observed in a monitored setting for approximately one hour following laparoscopy, after which patients are routinely discharged home. An assessment at the time of discharge will be recorded in study documentation and scanned into the electronic medical record.

The location and identity of each tumor will be recorded in study documents and scanned into the electronic medical record. The biopsy material will be sent to the pathology lab as part of standard treatment. A biopsy aliquot for experimental histologic or biomarker analysis will be retained in the investigators' laboratory.

3.1.3 Follow up and second standard-of-care surgery

A follow up telephone assessment will be made within 24 hours of discharge to monitor for any adverse events. This assessment will be recorded in study documentation and uploaded into the electronic medical record.

Participants will then undergo their second, planned, definitive operation within 14-30 days following injection. In addition to sending tumor material obtained during surgery for routine pathology assessment, the tumors injected with the interventional drug and the control solution will be removed and retained in the investigators' laboratory for biomarker analysis.

Per standard of care, patients will be followed clinically after their second, planned, definitive operations for a minimum of 30 days. Delayed adverse events will be monitored until 30 days following the second operation. Many patients undergoing definitive operations for abdominal metastatic cancer experience perioperative complications with predictable frequency. Therefore, only adverse events that cannot be reasonably attributed to the surgical procedure in the opinion of the physician investigator will be considered

possibly, likely or definitely related to the investigational agent according to the CTCAE attribution guidelines.

3.2 Allocation to Treatment Groups and Blinding

Not applicable.

3.3 Study Duration, Enrollment and Number of Sites

3.3.1 Duration of Study Participation

Total duration of the study will be up to 30 days, although patients will continue to be followed as part of standard clinical care for an additional 30 days following definitive surgery (tumor removal). No study interventions or study visits will occur during the 30 day post-operative period, although chart review and adverse event monitoring will continue per standard-of-care. Potential participants will be informed about the study. If interested in participating, they will be enrolled on the day of a standard-of-care laparoscopic surgery. Informed consent will be obtained for the research study as well as separately for the standard-of-care surgery.

3.3.2 Total Number of Study Sites/Total Number of Subjects Projected

The study will be conducted at AGH and WPH. Twelve subjects will be enrolled.

3.4 Study Population

3.4.1 Inclusion Criteria

1) Males or females age 18 to 99 years.

2) Pre-menopausal women ≥ 18 years of age must have a negative urine/serum pregnancy test prior to standard-of-care surgery and investigational treatment.

3) Participants must have an advanced intra-abdominal tumor, including metastatic or recurrent, biopsy-proven, digestive tract tumors.

4) Participants must have at least two index non-visceral intra-abdominal tumors that are grossly visible, >1cm^3 in volume, and amenable to biopsy and injection of investigational drug or control solution at the time of laparoscopy.

5) Participants must be planning or scheduled to undergo a standard-of-care abdominal laparoscopic surgical procedure at AGH or WPH and be potentially eligible for a second, definitive operation to remove the tumor(s) pending the findings during laparoscopy.

6) Must be able to read and understand English and consent for themselves.

3.4.2 Exclusion Criteria

1) Pregnant or lactating females

2) Investigational drug use within 30 days prior to enrollment.

3) Immunosuppressive medication including corticosteroids within 30 days prior to enrollment.

4) Active chemotherapy or radiotherapy within 4 weeks of investigational agent injection.

5) Active infection requiring systemic therapy or causing fever >38.1ºC or unexplained fever >38.1ºC within seven days prior to investigational agent injection

6) Laboratory abnormalities, drawn according to standard clinical care in anticipation of upcoming surgery outside the following limits:

AST/SGOT	> 1.5 times the upper limit of normal
ALT/SGPT	> 1.5 times the upper limit of normal
Total bilirubin	> 1.5 times the upper limit of normal
Creatinine	> 1.5 times the upper limit of normal
Hemoglobin	< 9 gm/dL
White blood cell count	< 3,000/ mm^3
Platelet count	< 70,000/mm^3
INR	>1.5 times the upper limit of normal
PTT	>1.5 times the upper limit of normal

7) History of allergic reaction to the investigational agent carrier solution.

8) Medical contra-indication or allergic reaction to acetaminophen or NSAIDs.

9) Participants who, in the opinion of the Investigator, may be non-compliant with study schedules or procedures.

10) Adverse events from prior therapy that have not resolved to CTCAE version 5 grade $\leq$1 prior to enrollment

Subjects that do not meet all of the enrollment criteria may not be enrolled. Any violations of these criteria must be reported in accordance with IRB Policies and Procedures.

4 STUDY PROCEDURES

4.1 Screening Visit

- Review of inclusion/exclusion criteria
- Review of demographics and medical history including allergies and medications
- Physical examination including vital signs (heart rate, blood pressure, temperature)
- Review of laboratory tests
- Review pregnancy test

- Explanation of study and provide informed consent document
- Complete study documentation and scan to electronic medical record

4.2 Study Treatment Phase

4.2.1 Visit 2 (at time of standard-of-care laparoscopic surgical procedure)

- Physical Exam including vital signs (heart rate, blood pressure, temperature)
- Review labs and medications and confirm eligibility
- Sign informed consent
- Standard-of-care surgical procedure including tumor biopsy
- Final intra-operative determination (based on surgical findings) of eligibility for second, definitive surgical procedure
- Injection of study drug and control solution into abdominal tumors (research procedures); record location
- Post-treatment assessment
- Complete study documentation and scan to electronic medical record

4.2.2 Visit 3 (telephone)

- Assess for adverse events related to study procedures
- Record use of acetaminophen or NSAIDs
- Record and classify any adverse events in the study documentation and electronic medical record

4.3 Final Visit (Second Standard-of-Care Surgery)

- Review interval history and medications (including acetaminophen and NSAIDs)
- Standard-of-care surgery including removal of tumors injected with study drug or control solution; tumors taken to investigators' laboratory for biomarker analysis
- Complete study documentation and scan to electronic medical record

4.4 Final Delayed Adverse Event Assessment

- Participants will be followed according to standard clinical practice for at least 30 days following second, definitive surgery and delayed adverse events will be

recorded via chart review during this period. However, no dedicated research procedures or study visits will occur.

4.5 Unscheduled Visits

Participants will be active patients of the study investigators, and will be seen on a same-day, ad hoc basis at the discretion of the participant and investigator in the event of an unscheduled need. The outcome of the visit will be recorded in the electronic medical record.

4.6 Concomitant Medication

Concomitant medications will continue uninterrupted at the discretion of the participant and the investigator. Concomitant medications will be recorded in the electronic medical record and reviewed at each study visit. Individuals who are scheduled to receive chemotherapy or immunosuppressive medications are not eligible for this protocol.

4.7 Rescue Medication Administration

Mild constitutional symptoms are anticipated based on prior studies. Over-the-counter anti-pyretic or anti-inflammatory medications can be taken at the discretion of the participant and the study investigator. Use of such over-the-counter medications will be assessed at each study visit.

4.8 Subject Completion/Withdrawal

Participants may withdraw from the study at any time without prejudice to their care. They may be discontinued from the study at the discretion of the Principal Investigator for lack of adherence to study treatment or visit schedules, or for adverse events. Investigator will record study completion, withdrawal or discontinuation in study documentation and in the electronic medical record.

4.8.1 Early Termination Study Visit

Early termination could theoretically happen if a participant is unable or unwilling to complete the second standard-of-care surgical operation within 30 days. In this unlikely scenario, a telephone visit would be utilized to assess for adverse events and to assess the reason for withdrawal or termination, and recorded in study documentation and the electronic medical record.

5 STUDY EVALUATIONS AND MEASUREMENTS

5.1 Screening and Monitoring Evaluations and Measurements

5.1.1 Medical Record Review

- Patient identifiers (MRN, date of birth)
- Sociodemographic variables (age, biologic sex, race, zip code of residence)
- Baseline physiologic variables (height, weight, BMI, BSA)

- Oncologic history (type of cancer, date of diagnosis, stage, treatment history, biopsy or pathology results)
- Comorbid conditions
- Medication review
- Allergy review
- Laboratory review
- Radiology review

5.1.2 Physical Examination

Physical examination will be performed per standard of care, to include general findings, vital signs, basic cardiopulmonary exam and abdominal exam.

5.1.3 Vital Signs

Vital signs are performed per clinic routine and include temperature, heart rate and blood pressure via automated measures. These will be done according to standard outpatient clinical practice.

5.1.4 Laboratory Evaluations

Standard-of-care laboratory studies, including pregnancy testing, will be reviewed but no labs will be drawn specifically for the study.

5.2 Efficacy Evaluations

5.2.1 Histologic and biomarker analysis

Tumor biopsy tissue will be subjected to standard-of-care pathology analysis according to hospital laboratory protocols. Assessment of biomarkers related to immune microenvironment will be performed in the investigators' research laboratories. Biomarkers of interest will include immunohistochemical and/or flow cytometry analysis to assess the nature of the immune cell infiltrate (e.g. cell lineage and activation status markers CD3, CD4, CD8, FoxP3, CD68); as well as proteomics analysis to analyze activation status of TLR4 and downstream transcription factors. The tissues will be kept indefinitely in the research laboratory of the principal investigator within AHN facilities. Access will be restricted to key study personnel. Genetic testing of the tissue is not planned. Results of the testing will not be made available to participants, nor placed in the electronic medical record. Although there are no plans to develop commercial products with this material, participants would not receive compensation or profits from any potential future developments.

5.3 Pharmacokinetic Evaluation

Not applicable.

5.4 Safety Evaluation

Safety evaluation will be performed at each study visit following the intervention, and continue throughout the 30 day standard-of-care perioperative period. This will include an interval history focused on symptoms or adverse events related to the study procedures, and use of acetaminophen or NSAIDs.

6 STATISTICAL CONSIDERATIONS

6.1 Primary Endpoint

The primary endpoint is to determine the feasibility, safety and tolerability of a pilot series of 12 patients undergoing injection of a bacterial-derived immunotherapeutic toll receptor agonist (LPS) instilled via direct injection into intra-abdominal tumors during laparoscopic surgery.

6.2 Secondary Endpoints

The secondary endpoint will be to assess whether discernable changes in biomarker correlates of the tumor immune microenvironment can be observed by comparing pre- and post- treatment standard-of-care biopsies.

6.3 Statistical Methods

6.3.1 Baseline Data

Descriptive analysis using proportions, mean and median values of sociodemographic and clinicopathologic variables will be presented.

6.3.2 Efficacy Analysis

Not applicable.

6.3.3 Pharmacokinetic Analysis

Not applicable.

6.3.4 Safety Analysis

The frequency of AE by type, body system, severity, and relationship to study drug will be summarized. Safety and feasibility failures will be characterized and reported. Significant adverse events (SAEs) will be reported promptly to the IRB and described in detail. AEs will be classified using the CTCAE, version 5.0.

6.4 Sample Size and Power

Sample size of 12 patients is justified based on common practice in early phase feasibility trials.

6.5 Interim Analysis

Not applicable.

7 STUDY MEDICATION (STUDY DEVICE OR OTHER STUDY INTERVENTION)

7.1 Description

The investigational drug is lipopolysaccharide (LPS) derived from *E. coli* 0113. LPS is the dominant component of gram-negative bacteria and has long been recognized as the principal chemical stimulus for sepsis during infections. In addition to stimulating systemic

toxicity, LPS is recognized directly by immune cells as a signal of infection through a cellular receptor known as Toll-like Receptor 4 (TLR4), which belongs to a family of pattern recognition receptors evolved to detect microbial material in human tissue. These receptors, when stimulated, result in activation of immune cells to express transcription factors and cytokines that alter the tumor immune microenvironment toward an adaptive anti-tumor state. The use of natural or synthetic molecules aimed at stimulating TLR4 and other pattern recognition receptors is an extremely active area of immunotherapy research. Intra-tumoral injection of LPS into abdominal tumors is a novel application of a concept that is otherwise in widespread use in cutaneous tumors including melanoma.

7.1.1 Packaging

The investigational drug will be obtained from List Laboratories, Inc., (https://listlabs.com/gmp-products/), who manufacture LPS products for research. According to the website and direct communication with the company, "This GMP compliant LPS drug product is available to clinical researchers with Investigational New Drug (IND) applications submitted to the Food and Drug Administration (FDA)." The product is packaged in 1μg vials and will be stored in the investigational pharmacy at AHNCI.

7.1.2 Labeling

The investigational drug is not currently labeled for individual use.

7.1.3 Dosing

Doses will be individually prepared per protocol by the investigational pharmacist. LPS obtained from List Laboratories, Inc., will be prepared sterilely at a concentration of 1ug/1mL and a dose of 1mL will be injected during laparoscopy.

7.1.4 Treatment Compliance and Adherence

All treatment doses are to be given in the operating room at WPH by a single physician investigator.

7.1.5 Drug Accountability

The investigational pharmacy and the principal investigator will be jointly responsible for maintaining the receipt and disposition records for study medications. Adequate records of study drug receipt and disposition will be maintained by the AHN Pharmacy Records of receipts, investigational drug orders, dispensing records, and disposition forms will be examined during the course of the study. The study medication is to be prescribed by the Investigator or designee and may not be used for any purpose other than that described in this protocol. All drug supplies including partially used and empty containers will be held in the investigational pharmacy.

8 SAFETY MANAGEMENT

8.1 Clinical Adverse Events

Clinical adverse events (AEs) will be monitored throughout the study.

8.2 Adverse Event Reporting

Based on prior studies with intravenous administration, the study drug is well tolerated with mild constitutional effects. Soft tissue intra-tumoral administration is anticipated to be less toxic than intravenous administration, and comparable to intra-dermal injection. Unanticipated problems related to the research involving risks to subjects or others that occur during the course of this study (including SAEs) will be reported to the IRB in accordance with Allegheny Health Network IRB SOP 011: Unanticipated Problems Involving Risks to Subjects. AEs that are not serious but that are notable and could involve risks to subjects will be summarized in narrative or other format and submitted to the IRB at the time of continuing review.

8.3 Definition of an Adverse Event

An adverse event is any untoward medical occurrence in a subject who has received an intervention (drug, biologic, or other intervention). The occurrence does not necessarily have to have a causal relationship with the treatment. An AE can therefore be any unfavorable or unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.

All AEs (including serious AEs) will be noted in the study records and on the case report form with a full description including the nature, date and time of onset, determination of non-serious versus serious, intensity (mild, moderate, severe), duration, causality, and outcome of the event.

8.4 Definition of a Serious Adverse Event (SAE)

An SAE is any adverse drug experience occurring at any dose that results in any of the following outcomes:

- death,
- a life-threatening event (at risk of death at the time of the event),
- requires inpatient hospitalization or prolongation of existing hospitalization,
- a persistent or significant disability/incapacity, or
- a congenital anomaly/birth defect in the offspring of a subject.

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug event when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

A distinction should be drawn between serious and severe AEs. A severe AE is a major event of its type. A severe AE does not necessarily need to be considered serious. For example, nausea which persists for several hours may be considered severe nausea, but would not be an SAE. On the other hand, a stroke that results in only a limited degree of disability may be considered a mild stroke, but would be an SAE.

8.4.1 Relationship of SAE to study drug or other intervention

The relationship of each SAE to the study intervention should be characterized using one of the following terms in accordance with CTCAE and Allegheny Health Network IRB Guidelines: definitely, probably, possibly, unlikely or unrelated.

8.5 IRB/IEC Notification of SAEs and Other Unanticipated Problems

The Investigator will promptly notify the IRB and FDA of all Suspected Unexpected Serious Adverse Events (SUSARs) that are related to the research activity. Other unanticipated problems related to the research involving risk to subjects or others will also be reported promptly. Written reports will be filed using the IRB system and in accordance with the timeline in the IRB SOP #011.

8.5.1 Follow-up report

If an SAE has not resolved at the time of the initial report and new information arises that changes the investigator's assessment of the event, a follow-up report including all relevant new or reassessed information (e.g., concomitant medication, medical history) should be submitted to the IRB and FDA. The investigator is responsible for ensuring that all SAE are followed until either resolved or stable.

8.6 Investigator Reporting of a Serious Adverse Event to Sponsor

Not applicable.

8.7 Medical Emergencies

Any medical emergency that arises during study drug dosing in the operating room will be handled per AHNCI policies and prudent medical practice in the judgment of the surgeon investigator.

9 STUDY ADMINISTRATION

9.1 Treatment Assignment Methods

9.1.1 Randomization

Not applicable.

9.1.2 Blinding

Not applicable.

9.1.3 Unblinding

Not applicable.

9.2 Data Collection and Management

Data collection will occur only at AHNCI facilities and will be maintained in a password-protected database, accessible only to key study personnel, on network servers that require institutional login access. Informed consent and study documentation will be scanned to this server and a hard copy retained by the principal investigator per institutional policy. For subsequent analysis and presentation, all protected health information will be removed from analytic datasets. A coded file linking study ID to patient identifiers will be retained on the password-protected institutional server and accessible only to key study personnel.

9.3 Confidentiality

All data and records generated during this study will be kept confidential in accordance with Institutional policies and HIPAA on subject privacy. The investigator and other site personnel will not use such data and records for any purpose other than conducting the study.

No identifiable data will be used for future study without first obtaining IRB approval. The investigator will obtain a data use agreement between the provider (the PI) of the data and any recipient researchers before sharing data.

9.4 Regulatory and Ethical Considerations

9.4.1 Data and Safety Monitoring Plan

This study will be overseen by the AHNCI Data Safety Monitoring Board (DSMB), which meets monthly to identify risks to research participants during the trial. An interim report to the DSMB will be made after the first three patients have been treated.

A staggered enrollment design will be followed in order to minimize the possibility of exposing subjects to serious adverse events. The staggering interval will be 21 days following the investigational drug agent to the third patient, which will be observed and reported to the DSMB prior to enrolling the fourth patient.

Pausing rules for this study will include death (other than death related to progressive disease) within 30 days of investigational agent exposure or $\geq$2 CTCAE version 5.0 grade 4 toxicities that are at least possibly related to the investigational agent. In either event, enrollment will be temporarily suspended pending assessment by the institutional DSMB.

9.4.2 Risk Assessment

Participants will incur greater than minimal risk in this study, related to the investigational agent. Risks related to standard-of-care surgery are not considered to be risks of the protocol or research procedures. Predictable risks of the investigational agent would be constitutional symptoms or vital sign abnormalities. These may be difficult to distinguish from vital sign abnormalities routinely encountered during and after laparoscopic surgery. Based on prior studies, adverse events are predicted to be mild and self-limited.

Patients in this study by definition will be undergoing concomitant treatment for advanced cancer and, as such, will be at risk for adverse events related to underlying disease or routine clinical care, including planned surgical procedures. These risks can involve serious adverse events that are unrelated to the study procedures and will be considered treatment-related when they cannot be reasonably attributed to standard-of-care procedures in the opinion of the principal investigator with oversight by the DSMB.

Risks of drug-related harm are minimized by (1) designing a test drug far lower than previously established tolerable doses calculated in prior studies examining intra-dermal injections of LPS; (2) selecting participants who will already have received pre-medication with anti-pyretic and anti-inflammatory medications as part of concomitant standard routine clinical care; (3) avoiding injections into visceral tumor or tumors that otherwise may bear close proximity to vital neurovascular structures; (4) employing a staggered enrollment design ensuring safety of the first three patients prior to exposing the next three patients to the investigational agent; (5) planned reporting to the DSMB following enrollment of the 3rd, 6th and 12th patients; and (6) observing study pausing/stopping rules in the event that SAEs are encountered during the study.

9.4.3 Potential Benefits of Trial Participation

Given that TLR4 agonists have shown significant promise as cancer therapeutic agents in pre-clinical studies, there is a potential direct benefit to advanced cancer patients in participating in a trial of this class of agents adapted to intra-abdominal delivery. There are indirect benefits as well- the novel process proposed here could allow other investigators to design similar intra-tumoral drug delivery strategies to anatomic locations previously beyond reach.

9.4.4 Risk-Benefit Assessment

Overall, the benefits both direct and indirect outweigh the risks of drug-related adverse events, which are anticipated to be mild and self-limited. Intra-tumoral treatment strategies represent a vital area of development for promising immunotherapeutic regimens. If successful, intra-abdominal intra-tumoral delivery would pave the way for additional investigation to the benefit of countless advanced cancer patients who have few good treatment options at this time.

9.5 Recruitment Strategy

Participants will be recruited from the active clinical practice of the investigators. The investigators will approach potential participants and explain the trial concept and invite participants to enroll if they are interested. No specific recruitment materials will be utilized. There is no need to collect data about potential participants in advance of enrollment beyond that involved in standard clinical care. There is no concern about the enrollment target of 12 patients given the frequency of this condition.

9.6 Informed Consent and HIPAA Authorization

Written informed consent will be obtained by physician investigators. This will occur in the clinic, in private consultation rooms. Participants will be provided a copy of the

consent and a plain language study summary and study flow chart. Given the study design, the participants will already be consenting for a standard-of-care surgery during the clinic visit. Investigators will allow the participant to review the study materials and take additional time to decide up to the time of standard-of-care surgery. Participants will be informed by the investigator regarding prior studies with TLR agonists and the constitutional side effects. Participants will be informed that enrollment is entirely voluntary and will not impact their standard-of-care treatment in any way. HIPAA Authorization is obtained as standard-of-care workflow in the clinic.

9.7 Payment to Subjects/Families

No compensation is planned for participation in this study.

10 PUBLICATION

Findings will be published in a peer-reviewed format by AHN authors.

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