Form Approved Through 6/30/2012 OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

# Grant Application

*Do not exceed character length restrictions indicated.*

**LEAVE BLANK—FOR PHS USE ONLY.**

| Type | Activity | Number |
|---|---|---|
| Review Group | | Formerly |
| Council/Board (Month, Year) | | Date Received |

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
Colonic epithelial cell stat3 and tumorigenesis

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
*(If "Yes," state number and title)*
Number: Title: ASCRS Research Foundation- Limited Project Grant

**3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR**

3a. NAME (Last, first, middle)
Name of Applicant

3b. DEGREE(S)
MD

3h. eRA Commons User Name

3c. POSITION TITLE
Assistant Professor

3d. MAILING ADDRESS *(Street, city, state, zip code)*
Mailing Address of applicant

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Surgery

3f. MAJOR SUBDIVISION
School of Medicine

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: FAX:

E-MAIL ADDRESS:

4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes

4a. Research Exempt ☐ No ☐ Yes If "Yes," Exemption No.

4b. Federal-Wide Assurance No.

4c. Clinical Trial ☐ No ☐ Yes

4d. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. Animal Welfare Assurance No. A3272-01

| 6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)* | | 7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD | | 8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT | |
|---|---|---|---|---|---|
| From | Through | 7a. Direct Costs ($) | 7b. Total Costs ($) | 8a. Direct Costs ($) | 8b. Total Costs ($) |
| 7/1/2012 | 6/30/2013 | $50,000 | $50,000 | $50,000 | $50,000 |

9. APPLICANT ORGANIZATION
Name: Johns Hopkins University
Address: 733 N. Broadway
Baltimore, MD 21205

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
52-0595110
DUNS NO. 00-191-0777 Cong. District 7th

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: First Name Last Name
Title: Grants Associate
Address: 733 N. Broadway, Suite 117
Baltimore, MD 21205-2196
Tel: FAX:
E-Mail:

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: First Name Last Name
Title: Grants Associate
Address: 733 N. Broadway, Suite 117
Baltimore, MD 21205-2196
Tel: FAX:
E-Mail:

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*

DATE 2/29/12

PROJECT SUMMARY (See instructions):

Sporadic colorectal cancer (CRC) is the second leading cause of death in the United States and, hence, is a major public health problem for which there are no clear preventive measures. Although colonic epithelial cell genetic and epigenetic alterations are inevitable in CRC, what triggers colonic epithelia cell oncogenic transformation is poorly understood. We found that Min (APC ) mice colonized with an human colonic commensal, enterotoxigenic Bacteriodes fragilis (ETBF) demonstrate marked colonic inflammation and rapid colon tumor formation dependent, in part, on a predominant and selective Th17 response with early activation of signal transducer and activator of transcription 3 (Stat3) in colonic epithelial cells and immune cells. These results support the hypothesis that the microbiota can precipitate specific mucosal signaling and immune responses important in early neoplastic changes in CRC. We hypothesize that colonic epithelial cell Stat3 is a key oncogenic transcriptional regulator that can coordinate epithelial and mucosal immune signaling, contribute to contribute to the host-bacterial interaction and ultimately serve as a critical link between colonic inflammation and tumorigenesis. The goal of this project is to define the role of colonic epithelial cell Stat3 in ETBF-infected mice using mice with a selective knockout of epithelial cell Stat3. The findings will lay the groundwork for novel mechanism-based interventional approaches to treating CRC based on inhibition of Stat3.

RELEVANCE (See instructions):

Colon cancer is the second leading cause of cancer death for women and men. Our data show that a newly recognized common human stool bacterium called enterotoxigenic Bacteroides fragilis (ETBF) triggers colon tumors in mice and that Bacteroides fragilis toxin (BFT) secreted by ETBF is essential to colon tumor development. This project will identify the colon epithelial cell receptor for BFT and identify how BFT secreted by ETBF strains induce different numbers of colon tumors. The project results will provide new

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

| **Project/Performance Site Primary Location** | | |
|---|---|---|
| Organizational Name: Johns Hopkins University | | |
| DUNS: 0019107770000 | | |
| Street 1: 733 N. Broadway | Street 2: | |
| City: Baltimore | County: | State: MD |
| Province: | Country: USA | Zip/Postal Code: 21205 |
| Project/Performance Site Congressional Districts: 7th | | |

| **Additional Project/Performance Site Location** | | |
|---|---|---|
| Organizational Name: | | |
| DUNS: | | |
| Street 1: | Street 2: | |
| City: | County: | State: |
| Province: | Country: | Zip/Postal Code: |
| Project/Performance Site Congressional Districts: | | |

SENIOR/KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Program Director(s)/Principal Investigator(s). List all other senior/key personnel in alphabetical order, last name first.

| Name | eRA Commons User Name | Organization | Role on Project |
|---|---|---|---|
| First Name MI<br>Last Name | | JHU | PI |

OTHER SIGNIFICANT CONTRIBUTORS

| Name | Organization | Role on Project |
|---|---|---|

**Human Embryonic Stem Cells** ☒ **No** ☐ **Yes**

**If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list:** http://stemcells.nih.gov/research/registry/eligibilityCriteria.asp. *Use continuation pages as needed.*

If a specific line cannot be referenced at this time, include a statement that one from the Registry will be used.

**Cell Line**

The name of the program director/principal investigator must be provided at the top of each printed page and each continuation page.

## RESEARCH GRANT

## TABLE OF CONTENTS



* Follow the page limits for these sections indicated in the application instructions, unless the Funding Opportunity Announcement specifies otherwise.

Program Director/Principal Investigator (Last, First, Middle): Last, First Name Middle Initial

| DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY | FROM 7/1/2012 | THROUGH 6/30/2013 |
|---|---|---|

List PERSONNEL *(Applicant organization only)*
Use Cal, Acad, or Summer to Enter Months Devoted to Project
Enter Dollar Amounts Requested *(omit cents)* for Salary Requested and Fringe Benefits

| NAME | ROLE ON PROJECT | Cal. Mnths | Acad. Mnths | Summer Mnths | INST.BASE SALARY | SALARY REQUESTED | FRINGE BENEFITS | TOTAL |
|---|---|---|---|---|---|---|---|---|
| First Name MI Last Name | PD/PI | 6 | | | | 0 | 0 | 0 |
| TBD | Research Associate | 6 | | | 28,560 | 14,280 | 5,069 | 19,349 |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| **SUBTOTALS** → | | | | | | 14,280 | 5,069 | 19,349 |

| | |
|---|---|
| CONSULTANT COSTS | |
| EQUIPMENT *(Itemize)* | |
| SUPPLIES *(Itemize by category)*<br>Research Supplies | 8,976 |
| TRAVEL | |
| INPATIENT CARE COSTS | |
| OUTPATIENT CARE COSTS | |
| ALTERATIONS AND RENOVATIONS *(Itemize by category)* | |
| OTHER EXPENSES *(Itemize by category)*<br>Animal Care and Feeding | 12,000 |

| | | |
|---|---|---|
| CONSORTIUM/CONTRACTUAL COSTS | DIRECT COSTS | |
| **SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD** *(Item 7a, Face Page)* | | $ 50,000 |
| CONSORTIUM/CONTRACTUAL COSTS | FACILITIES AND ADMINISTRATIVE COSTS | |
| **TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD** | | $ 50,000 |

## BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
## DIRECT COSTS ONLY

| BUDGET CATEGORY TOTALS | INITIAL BUDGET PERIOD *(from Form Page 4)* | 2nd ADDITIONAL YEAR OF SUPPORT REQUESTED | 3rd ADDITIONAL YEAR OF SUPPORT REQUESTED | 4th ADDITIONAL YEAR OF SUPPORT REQUESTED | 5th ADDITIONAL YEAR OF SUPPORT REQUESTED |
|---|---|---|---|---|---|
| PERSONNEL: *Salary and fringe benefits. Applicant organization only.* | 29,024 | | | | |
| CONSULTANT COSTS | | | | | |
| EQUIPMENT | | | | | |
| SUPPLIES | 8,976 | | | | |
| TRAVEL | | | | | |
| INPATIENT CARE COSTS | | | | | |
| OUTPATIENT CARE COSTS | | | | | |
| ALTERATIONS AND RENOVATIONS | | | | | |
| OTHER EXPENSES | 12,000 | | | | |
| DIRECT CONSORTIUM/ CONTRACTUAL COSTS | | | | | |
| **SUBTOTAL DIRECT COSTS** *(Sum = Item 8a, Face Page)* | 50,000 | | | | |
| F&A CONSORTIUM/ CONTRACTUAL COSTS | | | | | |
| **TOTAL DIRECT COSTS** | 50000 | | | | |

**TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD** **$** 50,000

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

First MI Last Name, PI (6 calendar months, no salary support) will be primarily responsible for this project. She will supervise and coordinate all aspects of the project. She is responsible for day-to-day design and execution of the experiments. She will review and interpretation of all data. She will be responsible for the writing of manuscripts resulting from the project.

Research Associated, (6 calendar months) will assist with mucosal immune cell isolation, PCR analysis, microbiology assays, immunohistochemistry.

Supplies ($8,976):PCR supplies[RNA extraction quantification as well as primers and probe for Taqman assays], immunohistochemistry/FISH[antibodies and probes] and FACS[antibodies and supplies for isolation of infiltrating colonic immune cells], microbiology supplies[bacterial culture plates and broth], scanning electron microscopy core usage.

Animal Care includes feeding and housing is estimated at $12,000 in year 1 (approximately 300 mice are required to complete the proposed experiments)

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

| NAME | POSITION TITLE |
|---|---|
| First MI Last, M.D. | Assistant Professor of Surgery |
| eRA COMMONS USER NAME (credential, e.g., agency login) | |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| Johns Hopkins University, Baltimore, MD | B.A. | 1995 | Biophysics |
| Vanderbilt University Medical School, Nashville, TN | M.D. | 2000 | |
| University of California, San Francisco, San Francisco, CA | Residency | 2000-7 | General Surgery |
| Cleveland Clinic, Cleveland, OH | Fellowship | 2007-8 | Colorectal Surgery |

### A. Personal Statement.

I am a board-certified colorectal surgeon with a focused clinical practice in colorectal cancer and inflammatory bowel diseases. My longterm goal is to begin to understand the early mechanisms of colorectal neoplasia and the relationship between the colonic flora and neoplasia. My career development award, under the mentorship of Dr. Cynthia Sears, focuses on the role of the immune system and signal transducer and activator of transcription (Stat) in colitis and, ultimately colon neoplasia. I am using the ETB-Min mouse model of colitis/colorectal cancer developed by Dr. Sears to study the role of Stats in colorectal carcinogenesis. In this model, tumor induction occurs, in part, via activation of colonic epithelial cell and immune cell Stat3, leading to a colonic mucosal immune response characterized by IL-17 production by Th17 cells. Thus, by using mice with selective knockout of Stat3 in the immune, epithelial and myeloid cell compartments, we are testing the hypothesis that Stat3 is a key oncogenic transcriptional regulator, a coordinate regulator of CEC and mucosal immune signaling that is critical to colonic inflammation and, ultimately, colon tumorigenesis. Ultimately, I am interested in translational applications of this work to patients with either sporadic colorectal cancer or inflammatory bowel disease associated colorectal cancer. In addition to my murine studies, I am collaborating with Dr. Sears to begin to characterize the the colonic flora and the tumor microenvironment in patients with colorectal cancer.

### B. Positions and Honors.

Employment

9/2008 Assistant Professor of Surgery
Johns Hopkins University School of Medicine

Other Experience and Professional Memberships

2010 American College of Surgeons – Fellow
2009 American Society of Colorectal Surgeons - member
Young Surgeons Committee (2008-11)
Program Committee (2010-13)
Quality and Safety Committee (2009-12)
Research Foundation (2011-present)
2008 Association for Academic Surgery – member
2003-4 University of California, San Francisco
Graduate Medical Education Council – surgical representative
2003 Society for Surgery of the Alimentary Tract – member

Honors

1994 Howard Hughes Summer Research Fellow, Johns Hopkins University

1995 Phi Beta Kappa, Johns Hopkins University
1996 Summer Research Fellowship, National Institutes of Health
1996 Exceptional Summer Student Award, National Institutes of Health
1997-8 Howard Hughes Medical Institute-National Institutes of Health Research Fellowship
2000 Surgical Clerkship Award, Vanderbilt Medical School
2003-4 Nafzigger Society Fellowship, University of California, San Francisco
2004 Outstanding Presentation Award; 17th Annual Resident Research Day, UCSF
2008 Canadian Society of Colorectal Surgeons Award for Best Presentation
American Society of Colorectal Surgeons (ASCRS) Tripartite Meeting

**C.Selected peer-reviewed publications (in chronological order).**


1. Park MK, Amichay D, Love P, **PI** Name et al. The CXC chemokine murine monokine induced by IFN-gamma (CXC chemokine ligand 9) is made by APCs, targets lymphocytes including activated B cells, and supports antibody responses to a bacterial pathogen in vivo. *J Immunol* 2002 Aug 1;169(3):1433-43
2. Hutter MM, **PI Name**, Lightner AM et al. Transient Receptor Potential Vanilloid Receptor–1 (TRPV-1) promotes neurogenic inflammation in the pancreas via activation of the Neurokinin 1 Receptor (NK1-R). *Pancreas*. 2005 Apr;30(3):260-5
3. **PI Name***, LaFleur S*, Imadulla P, Grady E, Bhargava A. Role of peripheral corticotropin-releasing factor and urocortin II in intestinal inflammation and motility in terminal ileum. *Proc Natl Acad Sci U S A*. 2005 May 24;102(21):7647-52 *co-first author
4. Lightner AM, **PI Name**, Jordan TH, Jaffray CE, Bunnett NW, Grady EF, Kirkwood KS. Neutral endopeptidase determines the severity of pancreatitis associated lung injury. *J Surg Res*.2005 Sep;128(1):21-7.
5. Cottrell GC, Roosterman D, Marvizon JC, Song B, PI Name et al. Localization of calcitonin receptor-like receptor and receptor activity modifying protein 1 in enteric neurons, dorsal root ganglia and spinal cord of the rat. *J Comp Neurol*. 2005 Sep 26;490(3):239-55.
6. **PI Name**, Pikios S, Grady EF, Bunnett NW and Kirkwood KS. CGRP partially mediates pain in acute pancreatitis. *Surgery.* 2006 Feb;139(2):197-201.
7. **PI Name**, Hoge, SG, Divino, LA, Grady EF, Bunnett NW and Kirkwood KS. TRPV-1,SP and CGRP mediate pain in acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol*. 2006 May;290(5):G959-69
8. Warne JP, Horneman HF, **PI Name**, Bhargava A, Pecoraro NC, Ginsberg AB, Akana SF, Dallman MF. Comparison of jugular versus superior mesenteric venous infusions of insulin in streptozotocin-diabetic rats on the choice of caloric intake, body weight and fat stores. *Endocrinology*. 2006 Nov;147(11):5443-51.
9. Finzi L, Barbu V, Burgel PR, Mergey M, Kirkwood KS, **PI Name**, Scoazec YV,Nordlinger B, Paye F, Nadel JA, Housset C. Overproduction of the gel-forming mucin MUC5AC in gallstone disease is triggered by an EGF-R pathway. *Am J Pathol*. 2006 Dec;169(6):2031-41.
10. **PI Name**, Gibbs L, Indorf LA, Varma M, Garcia-Aguilar J. Implementation of Quality Measures to Reduce Surgical Site Infection in Colorectal Patients. Dis Colon Rectum. 2008 Jul;51(7):1004-9. Epub 2008 Apr 16
11. Melton GB, Stocchi L, **PI Name**, Appau KA, Fazio VW. Contemporary surgical management for ileosigmoid fistulas in Crohn's disease. J Gastrointest Surg. 2009 May;13(5):839-45.

12. **PI Name**, Vogel JD, Church JM, Remzi F, Fazio VW. Surgical Site Infections (SSI) in a "High Outlier" Institution: Are Colorectal Surgeons to Blame? Dis Colon Rectum. 2009 Mar;52(3):374-9.
13. Wu, S. Rhee, K.J., Rabidzadeh, S., Housseau, F., Wu, X., Albesiano, E., Yen, HR., Huso, D., **PI Name**, Pardoll, D., Sears,CL. A human colonic commensal promotes colon carcinogenesis via activation of TH17 T cell response. *Nature Medicine*. Nat Med. 2009 Sep;15(9):1016-22.
14. Nathan H, Shore AD, Anders RA, **PI Name**, Gearhart SL, Pawlik TM Variation in Lymph Node Assessment After Colon Cancer Resection: Patient, Surgeon, Pathologist, or Hospital? J Gastrointest Surg. 2010 Dec 21. PMID: 21174232
15. **PI Name**, A Shore, K Hirose, S Gearhart, J Efron, J Vogel, J Clarke, M Makary Pay-for-Performance: A Disparity in Surgical Site Infection Risk and Cost for Obese Patients May Incentivize Surgeons Not to Treat. Arch Surg. 2011 May 16. PMID: 21576597
16. Hirose K, Shore AD, **PI Name**, Weiner JP, Makary MA. Pay for Obesity? Pay-for-Performance Metrics Neglect Increased Complication Rates and Cost for Obese Patients J Gastrointest Surg. 2011 May 1. PMID: 21533892
17. **PI Name**, A Shore, K Hirose, S Gearhart, J Efron, J Weiner, M Makary Readmission Rates and Cost Following Colorectal Surgery. Dis Colon Rectum. 2011 Dec;54(12):1475-9.PMID:22067174
18. **PI Name**, RE LeBlanc, G Ortega, C Robinson, E Platz, DM Pardoll, C Iacobuzio-Donahue, CL Sears. A shift from pStat6 to pStat3 predominance is associated with inflammatory bowel disease-associated dysplasia. Inflammatory Bowel Diseases. Inflamm Bowel Dis. 2011 Oct 21.

**D. Research Support.**

**Ongoing Research Support**

5 K08 DK87856-02 (Last Name) 07/01/2010 – 06/30/2015
NIH/NIDDK
Role of Stat3 in Colonic Inflammation
The goals of this project are 1: define in ETBF-infected mice, how Stat3 activation in distinct cell types(lymphocytes, colonic epithelial cells and myeloid cells) contributes to colitis and 2. Define mucosal immune profile (Th1/Th17 balance) in IBD patients who develop dysplasia and/or colitis associated cancer compared to controls.

90040916 (Wick) 07/01/2010 - 06/30/2012
American College of Surgeons (ACS)
The Role of Stat3 in Colonic Inflammation
This project will study how Stat3 activation in immune cells (lymphocytes and myeloid cells) promotes colitis in ETBF-infected mice.
No salary support for Dr. Last Name.

11117934 (Pronovost) 09/01/2011 – 08/31/2012
AHRQ
RFTO #5 Development and Demonstrations of a Surgical Unit-based Safety Program (SUSP) to Reduce Surgical Site Infections (SSI) and Other Surgical ComplicationsThis project will study the association between operating room culture and surgical outcomes including surgical site infection at 100 hospitals.

## Resources

**Sears Laboratory**
Refrigerators, shaking water bath, anareobic incubator, pH meter, sterile laminar flow hood, Forma ScientificCO2 water-jacketed incubator, analytical balance, two -20°C freezers, Lab-line Programmable Thermal Blok IIPCR machine, LKB Fraction collector, fast protein liquid chromatography (Pharmacia), gel electrophoresisequipment (Hoefer), Hoefer gel dryer, Ussingchambers adapted for use with cultured cells or intestinal tissue, two dedicated - 70°C freezers, light and phase microscopes, anaerobic hood.

**Pardoll Laboratory**
The lab is equipped with C02 incubators, laminar flow hoods, microscopes, table top centrifuges, water baths,chemical hoods and elctrophoresis supplies. Ultracentrifuge, scintillation counter, high speed centrifuge, HPLC, 7700 Sequence Detection System, dark room, -70C Revco freezer, liquid nitrogen freezer, cold room and iodination room are available as shared facilities. The division is fully equipped cold rooms (2), 37C room(1), two scintillation counters, three gamma counters, several ultracentrifuges and fluorescence microscope.

**Available in the CRB1, CRBII Buildings or Infectious Diseases Division for shared use**: Beckman gamma computer, Gilford spectrophotometer, Beckman Scintillation counter, a glassware room with autoclave, ultracold (Revco) freezer, ultracentrifuges, ice machines, sonicator equipment with microtip, Zeineh Scanning densitometer, LKB gradient high pressure liquid chromatography (HPLC), fluorometer, real-time PCR machines (iCycler, Cepheid, Light Cycler, ABI 7900HT), dark room, chemical fume hoods, iodination room, fluorescent microscopes, laser capture microscopy core facility. A flow cytometry center has been established within the Division of Molecular and Clinical Rhematology. The center is equipped with a dual laser FacStar puls and a FACScan flow cytometry machine. Each instrument is supported by a Hewlett-Packard computersystem and there exists a stand alone computer dedicated to data analysis.

**Johns Hopkins Core Resources**: Core laboratories providing protein and DNA sequencing, Affymetrix microarray facility, mass spectrometry, preparative protein analysis, mass spectrometry, microscopy analyses [electron microscopy (EM), immuno-EM, freeze-fracture, confocal, digital imaging, video microscopy, twophoton confocal, scanning EM, cryo-EM] and laboratory supplies are available to Dr. Sears' laboratory.

Additional details of Johns Hopkins Medical Institution Core Research Resources are available at: http://www.hopkinsmedicine.org/research/centers.html

**OTHER SUPPORT (Last Name)**
**ACTIVE**

5 K08 DK87856-02 (Last Name) 07/01/2010 – 06/30/2015 9.0 calendar mos. NIH/NIDDK
Role of Stat3 in Colonic Inflammation
The goals of this project are 1: define in ETBF-infected mice, how Stat3 activation in distinct cell types(lymphocytes, colonic epithelial cells and myeloid cells) contributes to colitis and 2. Define mucosal immune profile (Th1/Th17 balance) in IBD patients who develop dysplasia and/or colitis associated cancer compared to controls.

90040916 (Last Name) 07/01/2010 - 06/30/2012
American College of Surgeons (ACS) $40,000
The Role of Stat3 in Colonic Inflammation
This project will study how Stat3 activation in immune cells (lymphocytes, and myeloid cells) promotes colitis in ETBF-infected mice.
No salary support for Dr. Last Name.

11117934 (Pronovost) 09/01/2011 – 08/31/2012 0.6 calendar mos.
AHRQ $1,257,946
RFTO #5 Development and Demonstrations of a Surgical Unit-based Safety Program (SUSP) to Reduce Surgical Site Infections (SSI) and Other Surgical Complications
This project will study the association between operating room culture and surgical outcomes including surgical site infection at 100 hospitals.

**PENDING**

07/01/2012-06/30/2014
Kimmel Foundation Translational Scholar Award
Defining ETBF Induced Genetic Alterations in Colorectal Cancer
The goal of this project is to identify unique genetic changes in ETBF-induced tumors in mice.

**OVERLAP**

There is scientific overlap between K08 DK 087856 and ACS 90040916; 90040916 supports murine studies on the role of *STAT3* in ETBF induced colon carcinogenesis in immune cells.

**A. Hypothesis and Specific Aims:**

Sporadic colorectal cancer (CRC) is the second leading cause of death for men and women in the United States. The role of infectious and inflammatory processes in colon carcinogenesis is of intense interest since the colon is colonized with ~$10^{12\text{-}13}$ commensal bacteria with the potential to induce inflammatory processes if colonic epithelial homeostasis is disrupted. Drs. Sears and Pardoll, mentors to Dr. Last name, developed a system of infectious colitis by colonizing mice with a human colonic bacterium, enterotoxigenic *Bacteroides fragilis*
(ETBF), whose inflammatory properties are mediated, in part, by secretion of the metalloprotease toxin, BFT (*B. fragilis* toxin). Oral administration of ETBF to mice induces an acute followed by chronic colitis and a rapid (onset one week) and dramatic increase in colon tumor formation in multiple intestinal neoplasia ($Min^{Apc+/}$) mice. ETBF colonization rapidly and selectively activates Stat3 in both colonic epithelial (CEC) and inflammatory cells, leading to a selective intracolonic Th17 response. Colon tumorigenesis in this system is dependent on CD4+ T cells and IL-17, defining a novel pathway for T cell-dependent procarcinogenesis, in general, and a possible etiology for colon carcinogenesis specifically.

Tumorigenesis is also dependent, in part, on recruitment of the myeloid compartment and specifically, myeloid derived suppressor cells, to the tumor microenvironment. There is evidence for cell type dependent functions of Stat3 in intestinal homeostasis and pathology. Mice with a Stat3 KO in the myeloid compartment develop spontaneous colitis and mice with a Stat3 KO in the CD4 cell are resistant to autoimmune diseases. CEC Stat3 regulates expression of genes important for cellular proliferation, differentiation, apoptosis and wound healing and, in the azoxymethane and dextran sodium sulfate (AOM-DSS) model of colon carcinogenesis, mice with CEC Stat3 KO exhibit inhibited tumorigenesis.

Our general hypothesis is that Stat3, a key oncogenic transcriptional regulator, is a coordinate regulator of CEC and mucosal immune signaling and is critical to colonic inflammation and, ultimately, colon tumorigenesis. To begin to address this hypothesis, we are studying Min mice with selective KO of Stat3 in the colonic epithelial cell (villin cre Stat3 KO Min mice). Preliminary analysis of the ETBF-villin cre Stat3 KO Min mice at 1 week demonstrates that loss of CEC Stat3 results in more colonic inflammation and hyperplasia as compared to ETBF-Min mice. At 3 months, the ETBF-villin cre Stat3 KO Min mice have less colonic inflammation and tumors as compared to ETBF-Min mice suggesting that the CEC is playing a critical role in coordination of the inflammatory response and tumor progression. In the ETBF-Min mouse model, after inoculation with ETBF, mice are routinely colonized until the death of the mouse (typically 4 months after initial ETBF colonization). Surprisingly, after 2-3 months, the villin cre Stat3 KO Min mice either have significantly less or no fecal ETBF colonization suggesting that CEC Stat3 may contribute to ETBF colonization potential. We seek to further define the mechanisms by which Stat3 activation in the CEC contributes to ETBF colonization, colonic inflammation and ultimately tumorigenesis.

**Aim 1: To determine if CEC Stat3 is essential for maintenance of ETBF colonization.**

**Hypothesis 1:** CEC Stat3 is critical for maintenance of murine ETBF colonization. To begin to test this hypothesis, villin cre Stat3 KO Min mice and Min mice will be housed in the same cage (identical environments) and a detailed time course monitoring fecal and mucosal colonization will be analyzed to determine if under similar environmental conditions colonization is maintained at a high level in both the villin cre Stat3 KO Min and the Min mice.

**Aim 2: To investigate the mechanism(s) by which Stat3 activation in the epithelial cell coordinates colonic inflammation.**

**Hypothesis 2:** Stat3 activation in CECs is critical for the Th17 response and recruitment of the myeloid compartment in ETBF colitis leading to tumor initiation and promotion. To begin to test this hypothesis, a detailed time course at 1 and 6 weeks and 3-4 months comparing ETBF-infected villin cre Stat3 KO Min mice and ETBF-infected Min mice will be analyzed. At each time point, the inflammatory cell infiltration in the normal colon (1 and 6 week and 3-4 month time point) and tumors (3-4 month time point) will be evaluated to determine the influence of Stat3 in the CEC on the Th17/Th1 immune response balance, recruitment of the myeloid compartment and the colon tumor burden.

These studies will systematically dissect the role of CEC-specific Stat3 in colitis and bacterial colonization in the ETBF-Min mouse model of CRC and will begin to identify if CEC Stat3 is essential for tumorigenesis in this

model. The findings will lay the groundwork for novel mechanism-based interventional approaches to treating CRC based on inhibition of Stat3.

**B. Background and Significance:**

**The Role of Inflammation/Immunity in Neoplastic Transformation is Established but Incompletely Understood.**

Chronic inflammation and, specifically, infection-associated inflammatory processes, have been well documented to enhance carcinogenesis in the affected organs. In humans, chronic hepatitis due to HBV or HCV greatly increases the incidence of liver cancer while gastric ulcer disease due to *Helicobacter pylori* infection is a precursor of gastric cancer[12]. In murine models, increased cancer incidence is likewise found in experimental models of both infection-induced and noninfectious inflammation. Dissection of mechanisms of innate immunity using conditional knockout mice have demonstrated an important role for nuclear factor kappa-B (NFκ-B) signaling not only in the epithelial cells that are the target of transformation but also in the myeloid cell compartment that contributes to inflammation. NFκ-B signaling is central to virtually all innate and adaptive immune responses and transcriptionally activates many proinflammatory cytokines. The importance of proinflammatory processes in colonic carcinogenesis is emphasized by the dramatically lower incidence of tumor formation in Min mice when TLR signaling (resulting in NFκ-B activation) is abrogated[3]. Much, however, remains to be determined regarding which of the many NFκ-B driven inflammatory processes truly drives carcinogenesis, although in some systems, IL-6 has been implicated as an important player[4]. One of the key procarcinogenic signaling pathways induced by IL-6 is the Stat3 pathway that plays an important role in transcriptional activation of proliferative, anti-apoptotic and pro-angiogenic genes involved in cancer growth[5]. Stat3 signaling also organizes the immune microenvironment of tumors to block the generation of antitumor immune responses[6].

In contrast to the significant body of work implicating these innate immune processes in carcinogenesis, there is little information on the role of adaptive immunity, particularly T cell responses, in the promotion of cancer. Given the ability of T cell responses to generate antitumor responses, as well as the increased incidence of tumors in $Rag^{-/-}$ mice[7] (lacking T and B lymphocytes) and mice with defective interferon signaling[8], it has been postulated that chronic innate inflammatory responses promote carcinogenesis while T cell-dependent responses inhibit carcinogenesis. However, it is now clear that there are a number of qualitatively distinct types of T cell response characterized by distinct patterns of cytokine production. Three effector pathways of T cell differentiation have been defined – T helper 1 (Th1) responses promoted by Stat1 and Stat4 signaling, Th2 responses promoted by Stat6 signaling and Th17 responses promoted by Stat3 signaling. Th1 responses, driven by IL-12 and characterized by IFN-γ production, have been shown to be anti-carcinogenic in a number of systems while, until recently, little was known about the contribution of Th2 or Th17 responses to cancer[9]. Recent data revealed that human colorectal tumors associated with a Th17 response and/or Stat3 are associated with a worse prognosis[10-12]. Our observations regarding Th17-dependent colon tumorigenesis in ETBF-colonized Min mice (described in detail in Preliminary Data) form the basis for this proposal[13].

**ETBF: A Potent Microbial Inducer of Colonic Inflammation.** The role of infectious and inflammatory processes in colon carcinogenesis has been a matter of intense interest since the colon is colonized with ~$10^{12}$ commensal bacteria with the potential to induce inflammatory processes if colonic epithelial homeostasis is disrupted. Indeed, certain chronic inflammatory bowel diseases of the colon, particularly ulcerative colitis, are associated with the predictable development of colon cancer over time[14]. Although commensal bacteria are often cited as a critical environmental factor influencing the development of colorectal neoplasia, linkages to either specific organisms or groups of organisms, and the mechanisms promoting oncogenesis, have been tenuous[15, 16]. Supportive human epidemiologic data show that migrating populations adopt the cancer risk of

the region to which they relocate, observations proposed as due to dietary influences that alter the colonic microbiome, yielding an oncogenic environment in the colon. Despite considerable effort, no direct links between the metabolic activities of bacteria and colitis-associated cancer (CAC) or sporadic colorectal cancer (CRC) have been established. To date, the strongest, yet limited, evidence suggesting that commensal microbiota contribute to CRC pathogenesis derive from select, usually immune, gene knockout (KO) murine models in which the incidence of colon tumors is usually, but not always, diminished under germ-free conditions[17].

Enterotoxigenic *Bacteroides fragilis* (ETBF) are a molecular subgroup of *B. fragilis* that secrete a distinct, potent metalloprotease toxin (termed the *B. fragilis* toxin or BFT)[18]. BFT binds to a specific colonic epithelial cell (CEC) receptor triggering an array of CEC signal transduction mediated by NFκ-B, mitogen activated protein kinases (MAPKs), tyrosine kinases and β-catenin [19-21]. BFT further stimulates rapid cleavage of the tumor suppressor protein and intercellular adhesion protein, E-cadherin[22, 23]. Cleavage of E-cadherin yields increased mucosal permeability. Increased mucosal permeability, also a finding in IBD, leads to mucosal immune system exposure to luminal bacterial antigens and presumably activation of mucosal inflammation. E-cadherin loss in epithelial tumors is associated with enhanced metastatic potential and worse clinical outcomes[24]. In vitro, BFT activation of NFκ-B and β-catenin nuclear signaling leads to expression of proinflammatory cytokines by CECs and CEC proliferation [18, 20, 21]. In vivo, ETBF induce colitis and tumors in Min mice that is absolutely dependent on BFT expression. Taken together, studies on the mechanism of action of BFT strongly suggest it is an inflammatory and oncogenic bacterial toxin.

Clinically, ETBF are consistently associated worldwide with human diarrheal disease, both in children and adults[18]. Of note, multiple studies show that a significant portion of the normal human population without diarrhea (~4 to ~40% ) is colonized with ETBF. Field data show that ETBF induce human inflammatory diarrhea, similar to shigellosis[25]. However, it is unknown if chronic colonization with ETBF induces low level, clinically asymptomatic colonic inflammation. Two small studies suggest an association between ETBF and clinically active IBD[2627]. A single prospective cross-sectional epidemiologic study from Turkey reported that fecal ETBF was associated with CRC; fecal ETBF were isolated in 38% of 73 patients with sporadic CRC but only 12% of 59 sex-and age-matched concurrent controls ($P<0.01$)[28]. The Sears Laboratory has evaluated CRC specimens for ETBF using classic and molecular microbiology techniques and found that 36/46 (78%) tumors are positive for BFT. Control samples from patients undergoing screening colonoscopy are presently being evaluated to determine the prevalence in patients without colorectal pathology.

**The Stat3 Transcription Factor May Promote Colorectal Neoplasia.** Stat proteins are a family of transcription factors activated by cytokine receptor signaling and central to the regulation of immune responses. Stats are activated by tyrosine phosphorylation in the cytosol, which leads to dimerization and translocation to the cell nucleus. Development of each of the 3 established effector arms of T cell immunity is absolutely dependent on activation of specific Stats. Among the Stat proteins, Stat3 transduces signals from numerous growth factor and cytokine receptors, including the pro-oncogenic IL-6 receptor, and is constitutively activated in both inflammatory conditions and cancers, serving as a potential site of convergence for oncogenic signals[5]. Stat3 also transduces signals from the IL-10 family of cytokines (IL-10, IL-19, IL-22 and IL-24) which tend to promote anti-inflammatory responses[29]. In tumors, IL-6 mediated Stat3 activation promotes growth by up-regulating proliferation, cell survival genes, and angiogenesis genes, and enhances tumor immune evasion by inhibiting certain proinflammatory cytokines and chemokines. In the AOM-DSS model of colon carcinogenesis, myeloid cell derived IL-6 promotes tumorigenesis via activation of CEC Stat3[30]. In small studies, activated Stat3 (pStat3) has been identified in intestinal tissues of IBD patients and correlates with development of dysplasia and colitis associated cancer[31,32]. Activated Stat3 is also observed in human sporadic CRC where the amount of pStat3 correlated with the depth of tumor invasion[10, 33, 34].

Importantly, although Stat3 signaling has been shown to be absolutely required for Th17 cell generation, it may simultaneously serve as a negative regulator for Th1-mediated inflammation[35,36]. Similarly, in a model of carcinogen-induced skin cancer, IL-12, a promoter of Th1 responses, was anti-carcinogenic and IL-23, a

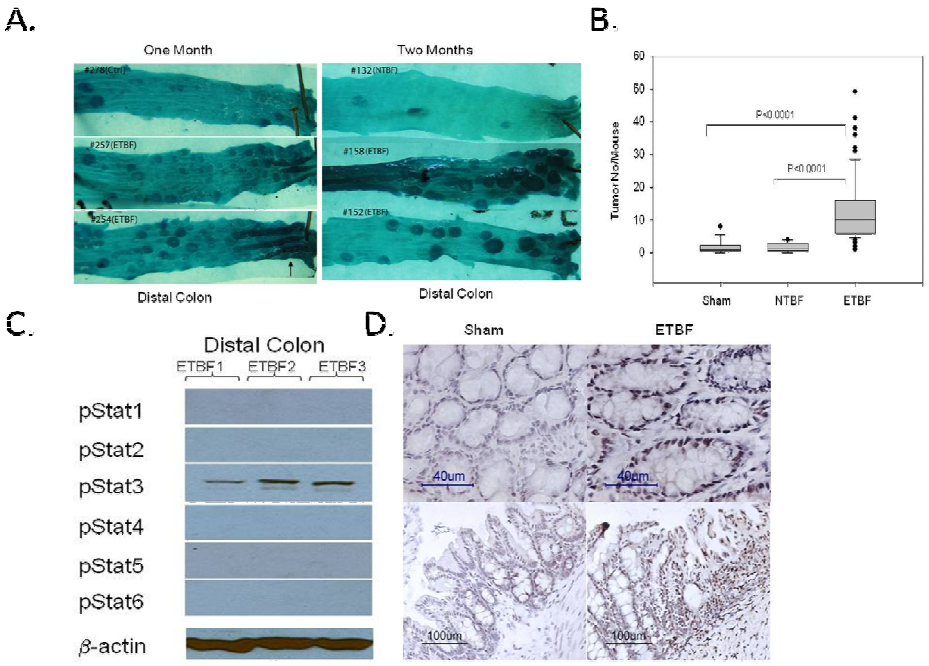
promoter of Th17 responses, was pro-carcinogenic, suggesting that Th1/Th17 immune balance is important in tumor pathogenesis[37]. Stat3 activation has been shown to inhibit IL-12p35 transcription and induce IL-23p19 transcription, suggesting that Stat3 signaling shifts the character of immune responses from anti-carcinogenic to pro-carcinogenic. CEC Stat3 controls transcription of IL-6 and IL-11 suggesting that CEC Stat3 KO could influence the overall Th1/Th17 immune balance in the tumor microenvironment[38].

**Summary:** The colonic flora is thought to contribute to both colitis and colon tumorigenesis, but the specific bacterial species and mechanisms have remained elusive. As presented in Section C, we demonstrate dramatic enhancement of colon tumor formation in Min mice colonized with the anaerobic human colonic bacterium, ETBF, a process dependent on Stat3/Th17 colon mucosal immune responses. ETBF-induced tumors are also marked by increased Stat3 expression in the CEC. Our preliminary results with ETBF-villin cre Stat3 KO Min mice demonstrates that deletion of CEC Stat3 leads to a lower tumor burden at 3 months but unexpectedly, also is associated with loss of ETBF colonization. Herein we propose to systematically dissect the role of CEC-specific Stat3 in the inflammation and bacterial colonization in the ETBF-Min mouse model of CRC and begin to identify if CEC Stat3 is essential for ETBF-induce tumorigenesis. The findings will lay the groundwork for novel mechanism-based interventional approaches to treating CRC based on inhibition of Stat3.

**C. Preliminary Results:**

**1. ETBF induces colitis and colon tumor formation in Min mice that is associated with Stat3 activation and a mucosal Th17 immune response**.

Min mice (Min$^{Apc+/-}$) develop intestinal tumors when the second copy of the *APC* gene spontaneously mutates.[39] Min mice mostly develop small bowel tumors beginning at ~3 months, and by 4-6 months, have developed usually <5 colon tumors per mouse. In contrast, Min mice colonized with ETBF, but not sham-inoculated or Min mice colonized with nontoxigenic *B. fragilis* (NTBF), develop chronic, asymptomatic colitis with intraepithelial colon tumor foci detected by one week. By one to two months after ETBF inoculation, ETBF colonization is associated with a marked increase in gross colonic tumor formation not observed in controls (Figure 1 A and B). Tumor induction and numbers correlate with microscopic colon hyperplasia and inflammation ($P \leq 0.003$) and occur predominantly in the distal colon, consistent with the distribution of human CRC.




A. Methylene-blue-stained representative samples of distal colons from Sham, NTBF and ETBF mice demonstrating excess tumors. Top panels, left (one month) sham and right (two months) NTBF. Bottom two panels for one and two months are ETBF. B. Tumor numbers at 4-6 weeks in Sham, NTBF and ETBF inoculated mice C.Western Blot analysis showing predominant Stat3 activation in colons of 3 ETBF colonized mice. D. IHC demonstrating increased pStat3 staining in distal colon of ETBF colonized mice 4 weeks after inoculation as compared to sham mice. Top panel colon CEC staining and a subset of inflammatory cells in the lamina propria of ETBF-colonized mice also exhibit pStat3 staining (arrow). Bottom panel on right shows an area of ETBF colon with Stat3 (+) immune cells

**Figure 1: ETBF enhances colonic tumor formation in Min Mice via Stat3 activation**

Small bowel tumors do not increase in ETBF-colonized Min mice, consistent with the known colonic niche for *B. fragilis*. These results suggest that ETBF rapidly induce de novo tumor formation and may serve as a tumor promoter.

To address mechanisms of ETBF-induced colitis and tumorigenesis, colonic mucosal activation of Stat proteins was assessed. When antibodies specific for each pStat protein were used, pStat3 was essentially the only activated Stat detected by western blot in nuclear extracts from the colonic mucosa of mice colonized with ETBF for 2 days, a time point at which colitis was already established (Figure 1 C). Minimal pStat4 signal was detected by western blot in a subset of ETBF-colonized Min mice. pStat3 was not detected in the colons of either sham or NTBF-colonized mice (data not shown). Immunohistochemistry (IHC) of colons from ETBF-colonized mice revealed pStat3 in both the CECs and a subset of infiltrating lymphocytes (Figure 1 D). Furthermore, pStat3 staining was intense in all colon tumors in ETBF-colonized mice (data not shown).

Conditional Stat3 knockout in CD4+ T cells in vitro and in vivo has revealed that Stat3 signaling is absolutely required to generate Th17 cells. Moreover, pStat3 is known to bind to the IL17 promoter.[40] A FACS (fluorescent activated cell sorter) analysis of isolated lamina propria cell infiltrates detected an expansion in CD4+ T cells in the lamina propria of ETBF-colonized Min mice (but not sham or NTBF controls). Furthermore,

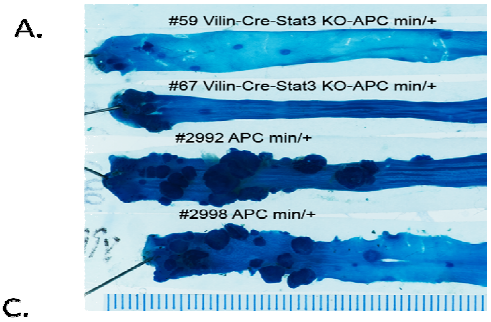
ETBF-colonized mice indeed developed a strongly skewed Th17 response characterized by predominant IL-17-secreting CD3+CD4+ and CD3+CD4- effector populations in the lamina propria. Further analysis demonstrated that the CD3+CD4- T cell population producing IL-17 is γδ-T cells. Intracellular IL-17 was also found in a subset of myeloid cells, myeloid derived suppressor cells (MDSCs).

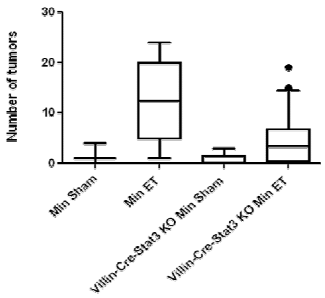
To specifically evaluate the contribution of the Stat3-driven Th17 inflammatory cells to ETBF-induced tumor formation in Min mice, IL-17A and/or the IL-23 receptor (IL-23R) was blocked using monoclonal antibodies. IL-23R was selected for blockade due to its key role in Th17 cell expansion. These results demonstrated inhibition of colon mucosal proliferation and gross colon tumors in ETBF-colonized mice treated with blocking IL-17/IL-23R antibodies compared to ETBF mice treated with isotype control antibodies.

**These results demonstrated for the first time a Stat3/Th17 pathway for inflammation-induced cancer by a common human commensal bacterium (ETBF). Thus, this microbially-induced model of colitis CRC provides an excellent opportunity to dissect the contribution of different compartments of pStat3 to colitis and inflammation-induced colon tumorigenesis.** Among available models of CRC, the ETBF-Min mouse model is attractive for further studies given that ETBF is a human commensal-pathogen (see Background) and, thus, the results have the potential to be relevant to human CRC.

**2. Colonic Epithelial Cell Stat3 Contributes to ETBF-induced Tumorigenesis.**

To address the role of CEC Stat3 in colon tumorigenesis, ETBF-colonized Min mice, we are using a conditional CEC Stat3 KO mouse [villin-Cre X Stat3flox/flox Min mice]. We compared tumor numbers between these mice and Min mice at 3 months after ETBF inoculation (Figure 2A & B). Gross colon tumors were significantly decreased in number and size in the ETBF-villin cre Stat3 KO Min mice. IHC for pStat3 confirmed the villin cre Stat3 KO Min mice are lacking Stat3 in the CEC and tumor cells (data not shown). Preliminary histopathologic analysis at 3 months demonstrated decreased inflammation and hyperplasia in the ETBF-villin cre Stat3 KO Min mice suggesting that CEC Stat3 is critical for tumor progression and colitis. Histopathologic analysis of the ETBF-villin cre Stat3 KO Min mice at 1 week demonstrates more inflammation and hyperplasia as compared to ETBF-Min mice. qRT-PCR suggests that the initial inflammatory response in the ETBF-villin cre Stat3 KO Min mice as compared to the ETBF-Min mice is shifted to a Th1 (increased IL-12) from a Th17 (increased IL-17, IL-21, IL-10) predominant response. Surprisingly, between 2 and 3 months the ETBF-villin cre Stat3 KO Min mice either have




C.

| Mouse | CFU (#/g) 1 Week | CFU (#/g) 6 Weeks | CFU (#/g) 3 Months |
|---|---|---|---|
| 1 | 2.3E+10 | | 0.0E+00 |
| 2 | | 5.42E+09 | 0.0E+00 |
| 3 | | | 0.0E+00 |
| 4 | | 2.5E+09 | 6.5E+05 |
| 5 | 3.2E+09 | | 2.3E+06 |
| 6 | 1.3E+10 | 2.6E+09 | 2.4E+06 |
| 7 | 2.9E+09 | 1.4E+08 | 3.5E+06 |
| 8 | 1.4E+10 | 3.2E+09 | 5.9E+06 |
| 9 | | 8.9E+08 | 3.1E+07 |
| 10 | 7.2E+09 | 7.0E+09 | 7.3E+07 |
| 11 | | | 1.6E+08 |
| 12 | 6.7E+09 | | 1.9E+08 |
| 13 | | 4.4E+09 | 2.6E+08 |

D.

| | Hyperplasia (Distal Colon)* Avg (SEM) | Inflammation (Distal Colon)** Avg (SEM) |
|---|---|---|
| ET-Min 1 Week | 1.33 (0.33) | 1.0 (0) |
| ET-Min VillinCre Stat3 1 week | 1.0 (0) | 1.67 (0.33) |
| ET-Min 3 months | 2.75 (0.25) | 2.75 (0.25) |
| ET-Min Villin Cre Stat3 3 months | 1.14 (0.14) | 2.14 (0.34) |

**Figure 2 Villin Cre Stat3 KO Min mice have diminished tumorigenenesis and inflammation**

A. Methylene-blue-stained representative samples of distal colons from ETBF-infected villin cre Stat3 KO Min and Min mice demonstrating diminished tumors. (3 months) panels for one and two months are ETBF. B. Tumor numbers at 3 months in Sham, and ETBF inoculated mice C. ETBF-villin cre Stat3 K0 Min have lower colonization at 3 months (ETBF-Min = $6x10^9$-$3x10^{10}$) D. Histopathologic scores from villin cre Stat3 KO Min and Min mice at 1 week (3 mice per group) and 3 months (7 mice per group) [scale 0-3: *normal to severe hyperplasia, ** normal to marked inflammation]

significantly less or no fecal ETBF colonization suggesting that CEC Stat3 may contribute to persistent bacterial colonization. The implications of the shifted immune response on tumor initiation are not known.

**These results suggest that CEC Stat3 may have multifaceted contributions to ETBF-induced colitis and tumorigenesis in Min mice including coordinating the immune response, contributing to bacterial colonization and promoting tumor progression. Herein, we propose experiments to begin to determine the influence of each contribution on tumorigenesis.**

**D. Research Design:**

**Aim 1: To determine if CEC Stat3 is essential for maintenance of ETBF colonization.**

**Hypothesis 1:** Stat3 activation in CECs is important for ETBF adhesion to the colonic mucosa. To begin to test this hypothesis, ETBF infected villin cre Stat3 KO Min and Min mice will be housed in the same cage and a detailed time course monitoring fecal colonization will be conducted to determine if, under similar environmental conditions fecal colonization in the villin cre Stat3 KO Min mice and the Min mice is constant ($10^9$-$10^{10}$ CFU/g).

**Rationale:** Murine colonization with ETBF is critical for ongoing colitis in the ETBF-Min mouse model. With persistent colonization, C57BL/6 mice (the background mouse for the Min mice) have intestinal inflammation and elevated levels of mucosal IL-17 up to one year after inoculation. At the point of colonization loss (12-18 months after inoculation), intestinal inflammation and mucosal IL-17 are not detected [levels are similar to sham C57BL/6 mice]. Together, these results suggest that ETBF infection must be ongoing to sustain intestinal inflammation and Th17 response. In contrast and surprisingly, between 2 and 3 months the villin cre Stat3 KO Min mice either have significantly less or no fecal colonization with ETBF suggesting that CEC Stat3 may contribute to bacterial colonization. Determining if colonization can be maintained in the villin cre Stat3 KO Min mice under stringent conditions is critical to establishing the role of CEC Stat3 in 1) bacterial colonization; 2) colonic inflammation; and 3) tumorigenesis in the ETBF-Min mouse model.

**Approach:** The basis of this specific aim is detailed monitoring of fecal ETBF colonization in the setting of co-habitation of villin cre Stat3 KO Min mice and Min mice. To ensure that villin cre Stat3 KO Min mice and Min mice are continually exposed to similar levels of environmental ETBF, all experiments will be conducted with mice from each group (ETBF-villin cre Stat3 KO Min and ETBF-Min) housed in the same cage. Mice routinely are re-inoculated during the course of infection as the result ETBF stool in the cage due to murine copropagic behavior. Extensive data from the Sears Laboratory indicates that ETBF-Min mice maintain fecal ETBF colonization at $10^9$-$10^{10}$ levels up to 3-4 months post inoculation. Unlike C57BL/6 ETBF mice, ETBF-Min mice only live for 3-4 months after inoculation because of bulky colon tumors causing bowel obstruction. Thus, our experimental design provides a stringent approach to test whether villin cre Stat3 KO Min mice are deficient in ETBF colonization potential.

**Mouse Inoculation and Monitoring**. To facilitate initial murine colonization with ETBF, we administer clindamycin (0.1 gm/L and streptomycin 5 gm/L) for 3-5 days before peroral inoculation of ETBF (~0.2 ml or ~$10^8$ bacteria in PBS) or PBS alone (sham control). When this approach is used, as in our Preliminary Data, ETBF (but not sham-inoculated controls) induce acute and persistent colitis in Min mice. Routine stool cultures reveal colonization rates on the order of >$10^{9\text{-}10}$ CFU/gm stool at days 7 and up to 3-4 months post-inoculation. To monitor villin cre Stat3 KO Min and Min ETBF infections, serial stool dilutions will be cultured anaerobically on selective nutrient agar to quantitate the inoculated strain (expressed as CFUs/gm stool). After inoculation, mice are also monitored daily by clinical criteria (e.g., activity, appearance, diarrhea, rectal bleeding [indicating onset of colitis]). Severely ill ETBF mice are euthanized (<5% of mice). Stool colonization levels will be evaluated weekly. Colonization levels greater than 1-2 log difference between the ETBF-villin cre Stat3 KO Min mice and ETBF-Min mice would suggest that the loss of colonization in the ETBF-villin cre Stat3 KO mice is intrinsic to the mice and not an environmental artifact, consistent with our hypothesis that CEC Stat3 is important to ETBF colonization in villin cre Stat3 KO Min mice. If no differences are detected between ETBF-

villin cre Stat3 KO Min mice and ETBF-Min mice, this suggests that the colonization defect identified in our initial experiments (when ETBF-villin cre Stat3 KO Min mice and ETBF-Min mice were housed in separate cages) can be overcome by repeated inoculations (ETBF-Min mice inoculating ETBF-villin cre Stat3 KO Min mice). We will include in our co-habitation experiment individual cages of ETBF-villin cre Stat3 KO Min mice and ETBF-Min mice as controls to confirm 1) persistent colonization in ETBF-Min mice and 2) loss of colonization in ETBF-villin cre Stat3 KO Min mice as seen in our preliminary data. In an initial experiment, the fecal colonization time course will be evaluated up to 3 months at which point colon tumors and ETBF-mucosal colonization will be evaluated (see next section). Each group will include at least 7-10 mice. We anticipate that all experiments will be repeated twice and sometimes three times given the biologic variability of the model.

**Analysis of Mucosal Bacterial Adherence.** At 3 months, the mice will be killed and colonic tumors will be counted. Quantitative measurements of bacterial adherence to the mucosa and structural studies of the relationship between the bacteria and epithelial cell will conducted on the colonic mucosa from the ETBF-villin cre Stat3 KO Min and ETBF-Min mice. Bacterial adherence is critical for enteric disease pathogenesis. Although available data suggest that *B. fragilis* adhere to the colon mucosa more avidly than other fecal flora, the adherence of *B. fragilis* to colon mucosa has not been carefully examined or quantified. We postulate that ETBF adhere more avidly (either qualitatively or quantitatively) to the Min colon mucosa as compared to the villin cre Stat3 KO Min mice as one explanation for the loss of fecal colonization in the villin cre Stat3 KO Min mice. Qualitative differences in the adherence of ETBF to the villin cre Stat3 KO Min colonic mucosa and the Min colonic mucosa will be assessed by histologic and ultrastructural examination of colon; quantitative differences by cultures of the mucosa and fluorescent in situ hybridization (FISH) for *B. fragilis*. To quantify adherence, duplicate pieces of proximal colon (~1 gm each) from each mouse will be washed thoroughly, homogenized and serial dilutions cultured anaerobically (expressed as CFU/gm tissue). This method detects both adherent *B. fragilis* and *B. fragilis* that may have invaded the mucosa. Colonic tumors will be counted and correlated with fecal colonization and qualitative and quantitative ETBF adherence results.

**Expected Results/Alternatives.** We anticipate that despite stringent conditions, ETBF-villin cre Stat3 KO Min mice will not be able to maintain colonization at 3 months suggesting that fecal and mucosal colonization are regulated by CEC Stat3. We predict mice with loss of colonization will have a lower tumor burden. With loss of fecal colonization, we expect that the mucosal colonization assay will demonstrate no mucosal ETBF. Alternatively, the stringent housing conditions may delay colonization loss and at 3 months, the ETBF-villin cre Stat3 KO Min mice will have similar or only slightly lower colonization (0.5-1 log difference) as compared to ETBF-Min mice. This may still suggest that the CEC Stat3 is important for ETBF colonization and repeat studies with a later time point (4 months) will be conducted to determine if the stringent environmental conditions simply delayed the loss. If loss is confirmed, we will seek to identify the earliest time point at which fecal colonization decreases to assess mucosal colonization at that time point and the interaction of the mucosa and ETBF as described. Further studies at the time point where loss is initially noted will focus on understanding the mechanism of loss (secreted antimicrobial peptides or anti-ETBF secreted IgA antibodies versus structural changes in ETBF-CEC relationship). In the event that colonization is not lost, our study will focus on the role of CEC Stat3 in tumorigenesis. With colonization intact, we anticipate that the ETBF-villin cre Stat3 KO Min mice will have similar tumor counts but the tumors will be smaller than in the ETBF-Min mice as a result of disruption of Stat3-mediated cellular proliferation pathways. Lastly, if loss of ETBF colonization occurs in the ETBF-villin cre Stat3 KO Min mice co-habitated with the ETBF-Min mice, it is possible that CEC Stat3 disrupts homeostasis with the murine microbiota such that a distinct microbiota emerges that is inhospitable to ETBF. To test this hypothesis, Illumina-based sequencing the microbiota of sham-villin cre Stat3 KO Min and sham-Min mice will be examine using fecal samples preserved in RNAlater.

**Aim 2: To investigate the mechanism(s) by which Stat3 activation in the epithelial cell coordinates colonic inflammation.**

**Hypothesis:** Stat3 activation in CECs is critical for ETBF colitis and tumor promotion. CEC Stat3 may be integral for initiation and/or maintenance of a Th17 response. To begin to test this hypothesis, a detailed time course at 1 and 6 weeks and 3 (or 4 pending the results of SA 1) months comparing ETBF-villin cre Stat3 KO Min mice and ETBF- Min mice will be analyzed. At each time point, the inflammatory cell infiltration in the

normal colon (1 and 6 week and 3 month time point) and tumor (3 month time point) will be evaluated to determine the influence of Stat3 in the CEC on the Th17/Th1 immune response balance, myeloid cell recruitment and the colon tumor burden.

**Rationale:** The Stat family of transcription factors are important mediators of cytokine signaling and coordinate innate and adaptive immune responses. Of the six family members, Stat1 and Stat2 regulate interferon responses and Th1 differentiation; Stat4, IL12-mediated Th1-cell expansion; Stat5, T-cell proliferation and erythropoiesis; and Stat6, Th2-cell differentiation. Stat3 plays multiple roles in innate and adaptive immunity, cell proliferation, apoptosis and differentiation. The role of T cell Stat3 signaling in Th17 differentiation and function is established. Increasing evidence suggests that Stat3 is important in the pathogenesis of both colitis and colorectal cancer. CEC Stat3 is required for IL-6 signaling, a cytokine which is considered a key coordinator of the Th17 response. We postulate that CEC Stat3 activation may be one of the key links between inflammation and cancer through promotion of a selective Th17-type colonic immune response. In ETBF-infected C57BL/6 mice, Stat3 activation is observed within 24 hours in the CEC, Stat3 activation persists in the CEC for up to 1 year but becomes patchy. In ETBF-Min mice, CECStat3 activation is also rapid (24 hours). Stat3 is prominent in the tumors. However, the specific contributions of CEC Stat3 to colonic mucosal inflammation are unknown. In ETBF-infected Min mice, IL-17 is critical for tumorigenesis and depletion of IL-17 abrogates tumorigenesis. Understanding how Stat3 activation contributes to colitis and the Th17 response and, ultimately, tumorigenesis may facilitate development of mechanism-based Stat3 specific therapies for CRC. Based on our preliminary data (histopathologic and qRT-PCR), at one week, the ETBF-villin cre Stat3 KO Min mice develop acute severe inflammation with mucosal ulcerations with increased IL-12 and decreased IL-17 as compared to the ETBF-Min mice, suggesting an acute Th1 as compared to Th17 response. Later time points have not been evaluated.

**Approach:**

**General Methods**

**Mouse Inoculation and Monitoring**. Mice will be inoculated at describe above in SA 1. Mice will monitored weekly to confirm ongoing fecal colonization given our preliminary data suggesting loss of fecal colonization in ETBF-villin cre Stat3 KO Min mice.

To define the contribution of CEC Stat3 to ETBF colitis, we will inoculate 7-10 mice per group per time point. Mice will be housed together. Mice will be studied at 1 and 6 weeks and 3 months after ETBF inoculation (or 4 months if fecal colonization is maintained in SA 1). These time points are based on our preliminary data in which both ETBF-colonized villin cre Stat3 KO Min and Min mice develop, by one week post-colonization, robust hyperplasia. In ETBF-Min mice, by 6 weeks, colon tumors are dramatically increased compared to the sham-inoculated group. The final time point will be 3 months (or 4 months if fecal colonization is maintained at this time point in SA 1). The later time point is selected based on the hypothesis that deletion of CEC Stat3 may delay but not prevent tumorigenesis.

**Analysis of Inoculated Mice:** As detailed below, ETBF-villin cre Stat3 KO Min or ETBF-Min mice will be analyzed to detect the impact of CEC Stat3 KO on the composition of the colon inflammatory infiltrates, expression patterns of key cytokines in the colon and tumors and alterations in balance of Stat proteins after ETBF inoculation. The results of SA 1 will guide our time point selections but we anticipate conducting experiments at 1 week, 4-6 weeks and 3-4 months. As in SA 1, mice will be co-housed. Each experimental group will have 7-10 mice.

**1. Analysis of colonic inflammation: FACS, qRT-PCR and IHC.** We will specifically determine if manipulation of CEC Stat3 expression shifts the immune balance (effector T cell [Th1/Th17], effector vs. regulatory T cell, recruitment of myeloid compartment) in ETBF-colonized colons. To accomplish this goal, as in our Preliminary Data, we will first analyze mucosal immune cells in the distal colon (1 and 6 weeks and 3-4 months) and tumors (3-4 months) by FACS. Second, we will selectively evaluate mucosal cytokine expression by qRT-PCR (distal colon and tumors). In initial experiments, colon inflammatory cells will be isolated. Initially the epithelial cell layer (colonic epithelial cells) will be isolated and then the remaining tissue will be digested (lamina propria cells). A percoll gradient will be used to separate the mononuclear cells. Cells will then be

stained first with surface staining antibodies and then fixed and permeabilized followed by staining for intracellular cytokines. FACS will be used to do subset analysis and determine if the relative composition of the inflammatory cells differs in ETBF-villin cre Stat3 KO Min mice vs. ETBF-Min mice. FACS allows us to further define the intracellular cytokine protein expression by specific immune cells isolated from the colonic mucosa, whereas qRT-PCR uses expression of cytokines for validation of intracellular cytokine staining as well as the opportunity to assay cytokines for which there are no good intracellular cytokine staining antibodies. For cytokines (unlike chemokines), mRNA detection (qRT-PCR) and protein detection serve as co-confirmatory methods. An exception is TGF-β where post-transcriptional processing is important for regulation. IHC will be used selectively to determine the relationship between key cell types in the tumor microenvironment (CD4, CD8, myeloid cells) providing the "geography" of cell type localization. It is now recognized that the location of specific immune cells (peritumoral vs. intratumoral) and proximity to other classes of immune cells may be important for certain functions.

FACS results will be analyzed together with qRT-PCR cytokine measurements in colitis tissue and tumors. This will allow us to further correlate CEC Stat3 activity with IL-17 intracellular expression (by FACS) and the expression of other cytokines (by Taqman qPCR) that either regulate Th17 immune responses (i.e., IL-1β, IL-6, IL-23p19, IL-27) or are coordinately produced by Th17 cells (i.e., IL-17A, IL-17F, IL-21 and IL-22). Of the several isoforms of IL-17, only IL-17A, C and F are expressed in humans and mice with IL-17A being predominant.

**2. Evaluation of Stat proteins.** As in our Preliminary Data, two approaches will be used to evaluate Stat activation in the colonic mucosa at 1 or 6 weeks or 3-4 months. Overall activation of Stats will be evaluated by western blot analysis using specific antibodies. We will also evaluate the activation of Stat3 (relevant to Th17 inflammation) and Stat1 and 4 (relevant to Th1 inflammation) to determine the effect of selective CEC Stat3 KO on the Stat-dependent signaling in ETBF-induced colitis and/or tumors. In some systems, Stat3 and Stat1 signaling are mutually antagonistic. This "yin-yang" relationship is particularly relevant in the context of Th1 vs Th17 responses (promoted by Stat1 and 4 versus Stat3 respectively) and carcinogenesis (thought to be enhanced by Stat3 signaling and inhibited by Stat1 signaling). We will also perform IHC for pStat1 and pStat3 to determine if protein detected by western blot is located in CEC or immune cells.

**Expected Results and Alternative Approaches:** We expect that composition of the inflammation will be altered in the ETBF-villin cre Stat3 KO Min as compared to ETBF-Min mice based on our preliminary experiments which demonstrated increased total inflammation and similar hyperplasia at 1 week and decreased inflammation and hyperplasia at 3 months by histopathology and a shift in Th1 versus Th17 cytokine expression at 1 week by qRT-PCR. However, we will extend these observations using FACS/qRT-PCR/IHC immunologic analyses and Stat western blots analyses to define if and how villin cre Stat3 KO modulates the immune response in the acute and chronic phases of ETBF colitis. The results of these studies will be correlated with tumorigenesis and bacterial colonization (SA1). Our preliminary observations (not shown in this proposal) indicate that in ETBF-Min mice Stat3 is activated first in mucosal immune cells (within 24 hours of infection) followed by marked CEC Stat3 activation (2-3 days). However, we do not know if the immune compartment Stat3 activation is the result of Stat3-dependent events in the CEC or is independent of a Stat3-mediated relay from the CEC which is triggered by ETBF through BFT (no inflammation or tumorigenesis occurs if Min mice are colonized with an ETBF strain in which BFT has been knocked out). Although CEC pStat3 cannot be visualized until 2-3 days after ETBF Min mice are infected with ETBF, it is possible that our detection by western blot or IHC is not sensitive enough to detect early, subtle CEC pStat3-dependent events. The villin Cre Stat3 KO Min mouse allows us to definitively identify the contribution of CEC Stat3 activation to the initiation and composition of the mucosal immune response to ETBF as well as subsequent colon tumorigenesis. These results hold tremendous promise to dissect the CEC molecular events triggered by a specific bacterial virulence factor that impact the type of mucosal immune response that ensues. CEC molecular events may be critical in differentiating immune responses (cell type and character) that are more likely to foster mucosal health (Th1) versus disease (Th17), particularly CRC

**Summary and Future Directions:** Our combined results from SA1 and SA2 are predicted to yield new insights into how Stat3 in the CEC contributes to a mouse's susceptibility to chronic colonization with ETBF,

composition of the microbiota, and the subsequent impact on mucosal inflammation and colonic disease. We hypothesize that the CEC is a coordinate regulator of the inflammatory and tumorigenic actions of ETBF since, to date, only CECs (or similar epithelial cells) express the BFT receptor. Our preliminary data suggest that loss of a single CEC molecule, Stat3, dramatically alters the outcome to ETBF infection. Hence this model provides the opportunity to dissect the molecular relay (microbiota:CEC:mucosal immune compartment) critical to colon tumor containment versus enhanced initiation and progression.

**E. Human Subjects.**

N/A

**F. Vertebrate Animals.**

Mice will be used for the experiments described in specific aim 1. All breeding will be done in the Johns Hopkins Cancer Center animal facility. The breeding and genotyping will be done by a laboratory technician dedicated to maintenance of Dr. Sears' mouse colonies. Based on our experience with the ETBF-induced colitis model, we anticipate needing at least 5-7 mice/condition with experiments repeated at least once, and usually twice, to allow for analysis of statistical significance between groups. In general, each experiment will consist of villin cre Stat3 KO Min mice and age and sex-matched litter mate control mice. Mice from each group will be inoculated with ETBF [and selectively PBS (sham)]. Mice will be inoculated at approximately 4 weeks of age. Experiments will be completed in either one (early) or six weeks or 3-4 months (late). Data analysis will follow. We anticipate the outlined experiments will require approximately 300 mice but numbers of mice will be adjusted based on experimental needs. Mice with ETBF colitis do not exhibit any outward signs of distress. Mice are euthanized by $CO_2$ asphyxiation before the intestinal tissue is obtained for experiments and histopathologic analysis. $CO_2$ asphyxiation is a method approved for euthanasia of mice by the Panel on Euthanasia of the American Veterinary Medical Association.

The mice will be housed in a brand new state-of-the-art facility in the same building as the research laboratories (CRB I and II). The Animal Care Facility is 100,000 square feet and employs 5 full-time veterinarians who are Diplomates of the American College of Laboratory Animal Medicine, as well as five additional veterinarians with training in laboratory animal medicine. Protocols for the experimental use of animals are reviewed by the Johns Hopkins University Animal Care and Use Committees. The animal care and use program at Johns Hopkins is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

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**H. Consortium/Contractual Arrangements.**
N/A

**I.Consultants.**
N/A

**J. Appendix.**
N/A