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