The Effects of Irgm1 on Intestinal Stem Cell Function

Introduction

Crohn's Disease (CD) is a chronic and potentially debilitating inflammatory bowel disease (IBD) that is characterized by overactive inflammation in any region of the alimentary tract, from mouth to anus. Numerous genetic factors, environmental triggers, and alterations within the gut microbiome have been identified as contributing to the etiology of CD (1). The current goal of CD treatment is to sustain patients in a state of clinical remission, staving off flares of intense inflammation that lead to hospitalizations, more severe immunosuppressive therapies, or surgical interventions. Many of the medications used to induce and maintain remission in patients with CD have the potential for serious side effects, including increased risks of cancer and infection (2, 3), due to their immunosuppressive nature. Such therapies also often fail to achieve lifelong remission. Accordingly, novel therapies that do not globally suppress the immune system are urgently needed. The overarching goal of Dr. Gulati's lab is to develop therapies that enhance intestinal epithelial cell function in patients with CD, thereby minimizing immunosuppressive side effects and promoting long-term intestinal healing. Intestinal stem cells (ISCs), as the drivers of intestinal epithelial turnover and differentiation, represent a key epithelial cell subtype that may be targeted to accomplish this goal.

To investigate the role of ISCs in CD pathogenesis, we will use a mouse model deficient for the protein, immunity-related GTPase family M member 1 (Irgm1). Irgm1 is a mouse homolog for human IRGM, which is a known risk allele for CD (4). Prior work by Dr. Gulati's group has established that *Irgm1* knockout (KO) mice display an increased susceptibility to intestinal inflammation in the small intestine and colon after exposure to the chemical trigger, dextran sodium sulfate (DSS) (5). Our intended use of the *Irgm1* KO model to study ISC dysfunction in a setting of chronic intestinal inflammation is based on two primary reasons. First, the observed CD-like ileocolonic inflammation in response to DSS was accompanied by abnormalities in Paneth cells, which are intercalated between ISCs within intestinal crypts and act as niche cells, providing regeneration-sustaining signaling to ISCs (6). Second, Irgm1 and IRGM play a prominent role in cellular autophagy (7), which is required for the destruction of many intracellular pathogens and for the recycling of intracellular components to be reused in times of cellular stress or starvation (8). We anticipate that the burst of epithelial proliferation exhibited by ISCs in response to injury will be diminished in the setting of diminished autophagic activity in *Irgm1* KO mice.

Despite these anticipated links between Irgm1 and ISC function, the exact regulatory effects of Irgm1 on ISC biology remain unclear. My project with Dr. Gulati will use the *Irgm1* KO mouse model to investigate the effects of IRGM on ISCs and their capacity for epithelial regeneration after radiation injury. Although we realize that radiation injury differs in many ways from the intestinal damage observed in CD, this approach represents a well-established model for studying ISC function, the central focus of this proposal.

Hypothesis

My hypothesis is that the *Irgm1* KO mice will exhibit diminished ISC function and regenerative potential in response to total body irradiation (TBI) compared to wild-type, irradiated controls.

Specific aims

1. Quantify the expression of particular mRNA transcripts thought to comprise markers of active stem cells (including Lgr5, Olfm4, and Ascl2) and of quiescent stem cells (including mTERT, HopX. and Bmi1) in *Irgm* KO vs. WT mice, with and without TBI. This distinction takes into account the two types of ISCs believed to reside in the gut—"active" vs. "reserve". While understanding that mRNA expression does not necessarily reflect the number of cells in each population, our assessment of the transcriptional responses of *Irgm1* KO mice to TBI should provide insight into how their ISCs are responding (or failing to respond) to this injury.
2. Assess in vivo ISC function in *Irgm1* KO vs. WT mice using an established microcolony assay protocol on histological sections after TBI. Briefly, we expect this assay, which quantitates the number of small intestinal crypts surviving post-TBI, to confirm a diminished ISC response and fewer surviving, regenerating crypts in *Irgm1* KO mice.

Background

Crohn's disease (CD) affects up to 700,000 patients in the United States alone, representing a significant cost to the healthcare system and placing a substantial, lifelong burden on affected individuals (9). Most of the current therapeutic options for CD involve intermittent or long-term immunosuppression, which contributes to an increased lifetime risk of cancer, a reduced capacity to fight infections, and a constellation of side effects with physical and psychosocial tolls. Although the etiology of CD remains unknown, recent reports have suggested that focusing on mucosal healing, rather than clinical endpoints alone, improves long-term outcomes in CD patients (10). An overarching goal of Dr. Gulati's lab is to develop immune-sparing therapies for CD, and my summer project will contribute to his investigation of mechanisms to optimize epithelial cell healing. In particular, the project will focus on intestinal stem cells (ISCs), which provide the driving force for the renewal and regeneration of all intestinal epithelial cell subtypes.

Within the crypts of the small intestine, crypt base columnar (CBC) cells represent the active population of ISCs that power the remarkable epithelial cell turnover in the normal intestine (11, 12). CBC cells express the intestinal stem cell markers, *Lgr5, Olfm4,* and *Ascl2* (13, 14). During normal homeostasis, a heterogeneous population of "reserve" ISCs can be found at the +4 position from the bottom of the crypt and express such markers as *mTERT, HopX,* and *Bmi1* (15). It is thought that these +4 cells play a key role in response to intestinal injury when they are recruited to maintain the epithelial turnover critical to intestinal health (16, 17). We believe that a better understanding of ISC biology in patients with CD could open up new pathways toward the augmentation of epithelial function in these individuals.

To investigate the activity of ISCs in epithelial regeneration after injury, we will use an *Irgm1*$^{-/-}$ mouse model that has an increased susceptibility to the development of ileocolitis, a common human CD presentation (5). Immunity-related GTPase family M member (IRGM) was identified as a risk allele for CD in a 2007 genome-wide association study (7), and subsequent studies have uncovered its critical role in cellular autophagy (8), participating in the destruction of intracellular pathogens and the recycling of nutrients in response to stress or starvation. While prior work with *Irgm1*$^{-/-}$ mice has confirmed the presence of dysfunctional antimicrobial peptide secretion in intestinal Paneth cells (5), the effect of *Irgm1* depletion on ISCs in this mouse model is currently unknown. In particular, after cellular injury, we anticipate that functional autophagy will be key to maintaining the proliferative demands of actively-replicating stem cells as they work to replenish the gut epithelium and may additionally affect the recruitment of the reserve +4 cells to action. Therefore, *Irgm1*$^{-/-}$ mice represent a novel model to study ISC activity and recruitment in a CD-relevant gene deficient system.

Actively dividing to renew the vast small intestinal epithelium, ISCs are acutely sensitive to radiation injury (18). Thus, total body irradiation (TBI) constitutes an excellent means to investigate the response of surviving ISCs after injury (19, 20). TBI marks the first step in the well-established microcolony assay, which has been standardized to facilitate the quantitation of functional intestinal crypts remaining after radiation injury. We expect that, at the same dose of radiation, epithelial renewal and stem cell activity will be more robust in WT mice than in autophagy-defective *Irgm1*$^{-/-}$ mice. Although TBI is clearly a different injury model than CD, it is an established tool to study ISC function. Therefore, applying this model to *Irgm1* $^{-/-}$ mice will refine our understanding of ISC responses in a CD-relevant knockout model. Clarifying these intestinal injury-response mechanisms will present novel approaches to optimizing the activity of epithelial ISCs and, ultimately, to achieving mucosal healing in patients with CD.

Research Design and Methods

Experimental Design: Adult, male C57BL/6 mice in specific pathogen-free (SPF) housing will be used for all experiments. Our four experimental groups are indicated in Table 1. Ten mice will be used for each group. All groups will be age- and gender-matched and will be derived from Het-breeding, such that WT and *Irgm1* KO littermates can be used. Before irradiation, the mice will be housed in groups of 4 in individually ventilated cages. Then, each mouse will receive either a 12 Gy dose of TBI using an X-RAD 320 irradiator (Groups 2 and 4) or a 0 Gy dose (Groups 1 and 3) to ensure equivalent exposure to stress among the four groups of animals. Post-irradiation, each mouse will be housed in a separate cage to reduce the potential influence of microbial exchanges before sacrifice at 3.5 days after irradiation. This time before sacrifice is dictated by the protocol of the microcolony assay to meet the 3-5 day window within which the intestinal epithelium is usually regenerated. Jejunal tissue for RNA extraction and histology will be collected at the time of sacrifice. Importantly, all tissues/specimens will be collected prior to my start date in the lab, thus ensuring the experiments described below will be feasible in the time frame of this proposal.

Table 1: *Experimental groups and expected outcomes*

Group	Treatment	Aim 1: expected expression of active ISC markers	Aim 2: expected microcolony assay findings
1: non-irradiated WT	0 Gy irradiation	Baseline	Normal # of crypts with baseline BrdU staining
2: irradiated WT	12 Gy irradiation	Increased	Fewer surviving crypts with increased BrdU staining
3: non-irradiated KO	0 Gy irradiation	? (close to baseline?)	? (Close to baseline # of crypts and BrdU staining)
4: irradiated KO	12 Gy irradiation	? (Increased, but lower than WT)	? (The fewest surviving crypts with increased BrdU staining)

Aim 1: *Quantify the expression of particular mRNA transcripts thought to comprise markers of active stem cells (including Lgr5, Olfm4, and Ascl2) and of quiescent stem cells (including mTERT, HopX, and Bmi1) in Irgm1 KO vs. WT mice, with and without TBI.*

Five mice from each group will be used for Aim 1. These numbers are based on the expected number of mice that will be available by this summer. I will use qRT-PCR to assess the levels of mRNA transcripts associated with “active” ISCs (Lgr5, Olfm4, and Ascl2) and with “quiescent” ISCs (mTERT, HopX, and Bmi1). RNA will be isolated from mouse jejunal tissue using a Qiagen RNeasy Kit (Hilden, Germany). Then, Taqman® (Applied Biosystems, Foster City, CA, USA) primer/probe sets for each of the 6 described transcripts will be used to assess the mRNA expression levels of each marker, based on its fluorescence relative to the housekeeping gene β-actin. The ΔΔCT method will be used to determine the fold changes in expression for each gene, normalized to β-actin, and relative to the baseline, non-irradiated WT group (Group 1).

Aim 2: *Assess in vivo ISC function in Irgm1 KO vs. WT mice using an established microcolony assay protocol on histological sections after TBI*

Five mice from each group will be used for Aim 2. These numbers are again based on the expected number of mice that will be available by this summer. Importantly, these 5 mice will have been injected with bromodeoxyuridine (BrdU) 1 hour before they were sacrificed.

Preparation of Histological Sections: This work will occur before my start in the lab. Paraffin-embedded jejunal sections from each mouse will be stained with H&E and immunohistochemistry with antibodies against BrdU. Briefly, 5-bromodeoxyuridine, which becomes BrdU upon its deamination and incorporation into the DNA of living tissues, is a thymidine analog; the applied anti-BrdU antibodies will highlight crypts with the rapid DNA synthesis characteristic of the surviving and actively proliferating ISCs. The use of BrdU allows for a more precise measurement of crypt survival compared to the more subjective evaluation of traditional markers of proliferation, such as high nuclear-to-cytoplasmic ratios, open nucleoli, and frequent mitotic figures.

Microcolony Assay: I will perform these analyses during my time in the lab. The microcolony assay allows for the assessment of stem cell survival via the quantification of the microcolonies (comprised of crypt-like cell foci) that are regenerated after acute stem cell injury. A well-established model of ISC injury is TBI. Within approximately three days of TBI, any surviving stem cells will have begun regenerating new crypt foci that can be then scored using a predictable and standardized system to assess relative crypt survival rates. Examples of surviving crypts are shown in Figure 1, although the images shown are not stained with BrdU, which will only enhance our ability to identify surviving crypts. Specifically, I will be quantifying the number of crypts from irradiated mice (Groups 2 and 4) with 5 or more BrdU-positive cells and then dividing this number by the total number of crypts per cross section in the 0-Gy controls (Groups 1 and 3). I will be blinded as to the group identity of each slide, and my assessments will be verified by a post-doctoral member of Dr. Gulati's lab with an extensive background in pathology.

Figure 1. *Sample WT and KO sections after a single dose of 12-Gy TBI*. In these preliminary H&E sections prepared by Dr. Gulati's lab, clear villus destruction (green arrow) and regions lacking actively-proliferating crypts (red arrow) can be seen in the KO mouse, compared to the heightened basophilic staining, indicating active DNA synthesis, seen more frequently in the actively proliferating crypts of the WT responding to injury.

Data Analysis

Aim 1: Fold changes in the mRNA expression of each of the active ISC markers (Lgr5, Olfm4, and Ascl2) and the quiescent ISC markers (mTERT, HopX, and Bmi1) will be calculated using the ΔΔCT method. These data will be assessed for normality. If normal, an ANOVA will be performed to compare means across all groups, followed by a Student's t-test for individual group comparisons (if the ANOVA is significant). If the data are found to be non-normally distributed, appropriate non-parametric testing will be performed.

Aim 2: The number of surviving crypts in each of the irradiated groups will be counted and divided by the number of crypts per cross-section in the 0-Gy controls (as described above). Statistical analysis will be identical to Aim 1.

Expected Outcomes and Alternative Approaches

Aim 1: I expect that the mRNA transcription of the actively cycling ISC markers (Lgr5, Olfm4, and Ascl2) will be decreased in KO mice compared to WT after radiation injury, although an overall increase or lack of change in active ISC marker expression might suggest that the +4 reserve ISC population has been recruited to make up for the injured CBCs. In such a situation, a decreased expression of quiescent markers may represent the activation (and subsequent decreased mTERT, HopX, and Bm1 expression) of +4 cells. A major drawback of using qRT-PCR alone is that only the relative expression levels, but not the localization of the transcripts or cell numbers, can be assessed. Prior work by Dr. Gulati's group has used the surface marker CD24 to identify ISC populations using flow cytometry (21). Indeed, once the preliminary data from this summer project has been collected and the relative levels of actively cycling ISC markers have been assessed, flow cytometry may provide greater insight into the dynamics of the ISCs in response to injury.

Aim 2: Regarding the functional microcolony assay, we expect KO mice to have relatively fewer surviving crypts than their WT counterparts after radiation, after controlling for the baseline crypt number in non-irradiated KO and WT mice, respectively. It is possible that the small number of mice available for each group will be insufficient to discern significant differences among the 4 groups; however, these preliminary data should at least provide a basis for useful analysis of variance and power calculations to better demonstrate significance in future work.

Works Cited

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Candidate's Statement

My primary goal for the summer is to gain experience using basic science techniques with future translational applications to pursue hypothesis-driven research. I also am particularly interested in researching Inflammatory Bowel Disease (IBD) and feel extremely fortunate to spend my summer amidst the many experts studying IBD at UNC. I have previously been exposed to clinical research strategies (studying the effect of obstructive sleep apnea on glaucoma progression) and qualitative research methods (conducting and analyzing interiews on the psychosocial impacts of receiving an IBD diagnosis during puberty). I approached this summer hoping to expose myself to translational and laboratory-based research on a topic about which I am passionate. At our first meeting, Dr. Gulati described how meaningfully his observations of his patients with IBD drive his work in the lab, and I believe his dual role as a clinician and a researcher will provide an optimal example of how translational research can contribute to bettering the lives of patients.

Having watched two of my closest friends and my brother grapple with IBD over the past decade, I particularly identified with Dr. Gulati's goal of developing non-immunosuppressive agents that promote mucosal healing and deep remission. One of the motivations behind the brief qualitative research project I completed during my master's work was rooted in the experience of my friend with ulcerative colitis. Her double-edged struggles with weariness from repeated steroid regimens and body image issues culminated in purposeful non-adherence to her maintenance pill in the hope of losing weight. Fear of prednisone-driven weight gain and moon facies development has characterized her experience with IBD. My brother's Crohn's has been well-managed by azathioprine, but knowledge of an increased risk of several cancers, including the usually-fatal hepatosplenic T-cell lymphoma, has been a dark cloud over his clinical remission.

Thus, in addition to my intellectual interest in IBD as a complex combination of immunology, microbiology, and chronic disease management with significant interdisciplinary ties to nutritional and psychological considerations, I would love to make real contributions to optimizing IBD treatment during my medical career for personal reasons. I look forward to honing my capability to carry out basic science techniques, to acquiring meaningful skill in analyzing pathological sections (which will help me in many potential medical paths), and to participating in journal clubs and lab meetings this summer. I believe my long-term goals will require an interdisciplinary skill set, and I am confident I could not be better-placed for my summer research project than in Dr. Gulati's lab.