The Effect of *Irgm1* on Intestinal Stem Cell Function

I. Background

Crohn’s Disease (CD), an inflammatory bowel disease characterized by areas of transmural inflammation anywhere in the GI tract (from mouth to anus) affects over 700,000 patients in the US alone, many of whom will require lifelong immunosuppression and/or surgical resection to maintain remission of their disease (1). CD pathogenesis is rooted in genetic susceptibility but requires an environmental trigger before establishing its pervasive, chronic GI inflammation. Genome-wide association studies have identified numerous risk CD risk alleles, many of which are related to the cellular recycling process known as autophagy, which is particularly important in stressful or nutrient-deficient settings (2,3). Among these genes is immunity-related GTPase M (IRGM); knocking out its murine homolog (*Irgm1*) generates increased susceptibility to ileocolitis (4). Moreover, in the intestinal epithelium, the Paneth cells of *Irgm1* KO mice have been shown to exhibit decreased functional innate immune-related defenses. Previous work suggests that dysfunctional Paneth cells may predispose intestinal dysbiosis (5).

In addition to regulating the intestinal microbiota, Paneth cells also play a role as niche cells for intestinal stem cells (ISCs). Given the improved outcomes among patients who are able to achieve epithelial healing as a clinical endpoint (6), Dr. Gulati’s lab is investigating whether ISC biology can be manipulated to optimize epithelial proliferation and, thereby, mucosal healing. Using mouse models lacking particular autophagy-related and CD susceptibility genes, such as *Irgm1*, the role of autophagy in the ISC milieu can be investigated. The intestine possesses two putative stem cell populations: 1) actively cycling Crypt Basement Columnar cells (CBCs) expressing such markers as *Lgr5*, *Olfm4*, and *Ascl2*; and 2) quiescent, “backup” stem cells at the “+4” position up from the base of the crypt, expressing such markers as *mTERT*, *HopX*, and *Bmi1*. In situations of injury or active stem cell depletion, “+4” cells are thought to be recruited to action in order to maintain the integrity of the intestinal epithelium (7).

Actively cycling stem cells bear the experimental boon of exquisite radiation sensitivity. Potten et al. (8) characterized these distinct populations and developed the “Microcolony Assay,” which utilizes total body irradiation (TBI) as the modality for investigating changes in the number of surviving crypts. While radiation enteritis is not intended as a faithful model of CD, the irradiation of *Irgm1* KO vs. WT mice should provide real insight into the role *Irgm1* plays in ISC biology. Further, preliminary work by the Gulati lab has demonstrated that *ex vivo* enteroid cultures from *Irgm1* KO mice show decreased proliferation and survival in culture, compared to WT enteroids. Enteroid systems are grown from actual intestinal crypts harvested from our mice of interest; when supplemented with a pro-proliferative medium, they reproducibly bud into enterospheres on day 1 and into functionally manipulable “mini-gut” systems by day 7 (9). Even though enteroids only comprise intestinal epithelial cells, these data suggest that the proposed *in vivo* model merits further exploration.

Hypothesis: *Irgm1* KO mice will exhibit diminished ISC function and regenerative potential in response to TBI, compared to wild-type controls.

II. Aims

1. Ascertain the effect of TBI on *Irgm1* transcription in the intestines of WT mice.

2. Compare the accuracy of qRT-PCR quantification of BrdU-labeled and non-BrdU-labeled intestinal tissues in WT and KO mice, with the hypothesis that BrdU labelling would alter the accuracy of qRT-PCR.

3. Quantify the mRNA transcription levels of active (*Lgr5*, *Olfm4*, and *Ascl2*) and quiescent (*mTERT*, *HopX*, and *Bmi1*) intestinal stem cell markers in *Irgm1* KO vs. WT littermate mice, with and without TBI treatment.

4. Assess the *in vivo* intestinal stem cell function in irradiated and non-irradiated *Irgm1* KO and WT littermate mice using the well-established microcolony assay protocol on BrdU-stained, paraffin-embedded slides.
III. Methods
Experimental Design: For these experiments, 8-12-week-old Irgm1 KO mice on a C57BL/6 background were matched by gender and age with WT littermates from heterozygous parents. All mice were housed in specific pathogen-free (SPF) conditions. At the time point of 3.5 days before sacrifice, which is the established timeframe to assess crypt survival (8), matched groups of 5-7 WT or KO mice received a 0 Gy or 14 Gy dose of TBI via an X-RAD 320 irradiator. The 14 Gy dose was selected based on previous experiments in the Gulati lab suggesting that this dose led to clinical differences in weight between WT and KO mice at certain time points. After irradiation, each mouse was individually housed and weighed daily.

Quantitative RT-PCR using jejunal and ileal tissues: At the time of sacrifice, jejunal and ileal tissue samples were stored in RNAlater for each of the 4 groups previously described: WT 0 Gy, WT 14 Gy, KO 0 Gy, and KO 14 Gy (n=5-7 mice/group). Using a Qiagen RNeasy Kit (Hilden, Germany), RNA was isolated from all tissues, and cDNA was produced. Quantitative RT-PCR was performed using Taqman® (Applied Biosystems, Foster City, CA, USA) primer/probe sets for active ISC markers (Lgr5, Olfm4, Ascl2), quiescent ISC markers (mTERT, HopX, Bmi1), and Irgm1. Data were analyzed using the ΔΔCt method, utilizing β-actin as the internal housekeeping gene control and WT 0 Gy mice as the baseline control group. Irgm1 transcription levels were measured in WT tissues from BrdU- and non-BrdU-treated mice.

Microcolony Assay (MCA): Two hours before sacrifice, WT and KO animals (n=5-7/group) from the same irradiated and non-irradiated groups described above received intra-peritoneal injections of BrdU, a thymidine analog that is preferentially incorporated within rapidly renewing cells. Paraffin-embedded jejunal and ileal sections from each mouse were immunohistochemically treated with antibodies against BrdU (UNC Histopathology Core), identifying replicating cells and facilitating a more precise tabulation of regenerating crypts for the MCA. All crypts containing 5 or more BrdU+ cells were counted, and the number of surviving crypts after irradiation was compared to the average number of proliferating crypts in WT and KO mice before treatment.

IV. Results

Figure 1: Intestinal Irgm1 expression is induced in response to TBI in a location-dependent manner. Data are normalized to β-actin and shown as means ± SD. WT mice with no TBI were used as controls. *p<0.05; **p<0.01.

1. Ileal, but not jejunal, Irgm1 expression is induced in response to TBI. Groups (n=5-6) of WT mice received either 0 Gy or 14 Gy of radiation, and RNA was isolated from whole jejunal and ileal tissues for qRT-PCR using Taqman Irgm1 primer/probe sets. Irgm1 KO mice at 0 and 14 Gy (n=5-7) were included as controls and all failed to amplify Irgm1 (data not shown). No significant difference in expression was noted in the jejunal tissues, but a roughly 4-fold increase expression post-TBI was found in the ileum.

Figure 2. BrdU labeling has an unpredictable effect on the efficiency of the qRT-PCR reaction. Data are normalized to β-actin and shown as means ± SD. Unlabeled, WT mice with no TBI were used as controls. *p<0.05; **p<0.01.

2. Significant differences in Irgm1 expression were noted in BrdU-labeled versus non-labeled tissues. Using cDNA that was made during the same PCR reactions as for the non-BrdU samples, 5 BrdU+ WT 0 Gy samples and 4 BrdU+ WT 14 Gy samples from both the jejunum and the ileum were used to investigate the effects of BrdU labelling on RT-PCR efficiency for both Irgm1 and β-actin (not shown). While a significant difference effected by BrdU labeling was only noted in jejunal WT tissues at 14 Gy, trends (p<0.15) toward differences were observed in jejunal 0 Gy and WT 14 Gy tissues, as well as in all groups probed for β-actin transcription. These trends were not uniformly towards increased or decreased transcription levels. Based on these data, we chose to run all further qRT-PCR assays using only non-BrdU-treated tissues.
Figure 3: TBI reduces the transcription of actively cycling ISC markers, with some changes based on anatomical location, and has even more pronounced location-specific effects on “quiescent” ISC markers in WT and KO mice. Data are normalized to β-actin and shown as means ± SD. WT mice with no TBI were used as controls. *p<0.05; **p<0.01.

3a-c. TBI decreased the mRNA transcription of actively cycling ISC markers in both the jejunal and ileal tissues, with minimal effect of Irgm1 on this process. As expected, all actively cycling ISC genes were less transcribed post-TBI in all tissues. Of note, Lgr5 expression was more significantly downregulated in KO mice compared to WT, and a significant difference was noted in ileal Lgr5 expression between KO and WT mice at 14 Gy. Interestingly, Ascl2 expression was significantly elevated at baseline in the jejunal tissues of KO vs. WT mice, with a similar trend in the ileum. Olfm4 expression trended towards a baseline elevation at 0 Gy in KO vs. WT mice, although no differences were found post-TBI in either tissue.

3d-f. TBI increased the mRNA transcription of quiescent ISC markers in the ileal tissues of WT and KO mice but had various effects on transcription in jejunal tissues. In ileal tissues, all quiescent ISC genes trended toward increased transcription in both WT and KO mice. Ileal Bmi1 expression was significantly increased post-TBI in WT vs. KO mice, and WT mTERT expression in the ileum was significantly higher only in WT, not KO, mice. In jejunal tissues, KO and WT mice demonstrated upregulated Bmi1 and HopX expression. KO mice had significantly lower levels of jejunal HopX expression at baseline. KO mice also demonstrated a more significant increase in Bmi1 expression than WT mice post-TBI. Uniquely, mTERT expression exhibited distinct tissue-specific trends, significantly decreasing in both WT and KO jejunal tissues while increasing in ileal tissues (significantly in WT mice, but not in KO) post-TBI.
V. Discussion

In humans, the most common inflammatory patterns of CD are ileitis and colitis. That these two regions are more microbiologically and immunologically complex than much of the rest of gut is unlikely to be a coincidence, particularly given the necessity for both genetic predisposition and environmental trigger in the pathogenesis of CD. That our Irgm1 KO mouse develops ileocolitis post-insult makes it an excellent CD model. Thus, the 4-fold upregulation of Irgm1 activity in the ileal, but not jejunal, tissues of WT (Figure 1) mice post-TBI seems to support a connection between Irgm1 transcription and ISC function. It is surprising that such a pronounced gradient exists between two neighboring sections of the small intestine; further investigation into gradients of Irgm1 expression may be interesting.

Due to the proposed role of Irgm1 in the ISC function, we expected that the loss of Irgm1 function would result in a more pronounced decrease in the expression of actively cycling ISC markers, Lgr5, Ascl2, and Olfm4, after ISC damage via TBI. Yan et al. showed that, in response to TBI, Lgr5 is depleted in murine intestinal tissue (10). The present results support these suppositions, as Lgr5 downregulation was noted in both jejunal and ileal tissues post-TBI. Knocking out Irgm1 produced a significant decrease in Lgr5 expression in the ileal tissues of KO, but not WT, mice; in jejunal tissues, a higher level of significance in decreased Lgr5 expression was met in KO, compared to WT, mice. Both Olfm4 and Ascl2 trended toward a baseline increased expression in KO mice, although this elevation was only significant in jejunal Ascl2 expression. Location within the small intestine seemed particularly important regarding Ascl2 expression, as KO jejunal tissues exhibited a significant decrease in response to TBI that was not found in WT tissues. No significant differences in Ascl2 expression related to phenotype or TBI were noted in ileal tissues. Similar to Lgr5, Olfm4 expression was more significantly diminished post-TBI in KO than WT mice in both jejunal and ileal tissues. However, so profound was the effect of TBI that no differences were noted between the KO and WT mice at 14 Gy.

As markers of actively cycling stem cells became depleted, we speculated that the markers of the quiescent, “+4” cell populations would be upregulated, driving epithelial regeneration more efficiently in WT than in KO mice. Yan et al., in compliment to their Lgr5 findings, showed that Bmi1 expression was enhanced post-TBI in WT mice (10). Our data, particularly from the ileum, support these hypotheses. Bmi1 expression was significantly upregulated in the ileal tissues of WT mice post-TBI, but KO mice showed hardly any upregulation, strongly suggesting a defect in the KO mouse’s ability to recruit “+4” cells in the setting of CBC depletion and injury. In the jejunum, however, both KO and WT mice had similarly increased expression of Bmi1 post-TBI, with mild significance (p=0.043) attained by the KO mice in response to TBI. Given our previous finding that Irgm1 expression is induced in response to TBI in the ileum, but not the jejunum, a role for functional Irgm1 in +4 cell recruitment may be supported by this deficient capacity to increase the proliferation of “+4” cells in KO mice, compared to WT mice.

As for the other quiescent ISC markers, less obvious differences in KO and WT function were noted, although our putative tissue specificity in response mechanisms to TBI was still supported. Perhaps mirroring the baseline elevations seen in Ascl2 and Olfm4 in the jejunum, the baseline jejunal expression of HopX was significantly lower.
in KO compared to WT mice. However, TBI produced no significant differences in HopX expression in either jejunum or ileum, though both tissues exhibited trends toward increased HopX expression post-TBI consistent with the Bmi1 findings. Ileal mTERT expression also followed a trend towards increase post-TBI, with no differences between WT and KO mice (that WT mice, but not KO, exhibited a significant upregulation of mTERT post-TBI is more likely a consequence of the larger variability of the KO ileal tissues sampled than a real diminished effect in the KO tissues). However, in jejunal tissues, both WT and KO mice showed significantly downregulated mTERT expression levels in response to TBI, completely the opposite of the ileal pattern. The expression of mTERT in response to TBI thus seems to be the most tissue-dependent of the 6 genes quantified here; however, we found little to suggest that Irgm1 plays much of a role in this expressional switch.

Overall, these data support similar responses to TBI among the quantified actively cycling ISC genes but a failure in KO mice to induce Bmi1 expression in ileal tissues. It is notable that, more strikingly in the jejunum, two of the actively cycling genes, Ascl2 and Olfm4, were elevated at baseline in KO mice compared to WT, and that HopX expression was decreased at baseline in KO mice compared to WT. Furthermore, tissue-specific patterns of response to TBI, and perhaps tissue-specific needs for functional Irgm1 in response to radiation injury, are supported by the different patterns in the Lgr5, mTERT, HopX, and Bmi1 jejunal and ileal expression data. Further investigation of ileal-distinct patterns to epithelial injury may better elucidate mechanisms of CD pathogenesis. The present data, obtained using a mouse KO model for a human CD risk allele, suggest that an Irgm1-requiring defect in response to epithelial injury could be implicated in the ileocolitis observed in these KO mice and in the epidemiological finding that ileitis and colitis are the two most common CD presentations. Unpublished discussions with collaborators have also suggested more damage is seen in the ileal tissues of WT mice and other mouse models, post-TBI. Future investigations into less common CD presentations, such as jejunitis alone, may identify novel patient group-specific therapies and/or highlight potential shared etiologies of tissue-specific CD. At least in mice, Irgm1 functionality does not seem to participate in any pan-tissue, common CD pathogenesis.

These qRT-PCR data were functionally assessed by the Microcolony Assay, which was performed on both jejunal and ileal tissues. Both WT and KO tissues demonstrated significant crypt drop-out post-TBI. A significant increase in crypt numbers was seen at baseline in KO vs. WT jejunal tissues, perhaps in line with the enhanced expression of Ascl2 and Olfm4 in these animals. However, these differences did not persist post-TBI. However, though lacking any baseline differences, ileal sections from KO mice showed enhanced crypt survival post-TBI, compared to those from WT mice. This result seems surprising, given our qRT-PCR data showing a decreased capacity to enhance Bmi1 expression after TBI in the KO animals. Interestingly, since this project was proposed, Sun et al. showed that the Irgm1 KO mouse demonstrates baseline enhanced epithelial turnover due to its elevated circulating Type I Interferons (11). These authors proposed a Wnt-independent, macrophage-transduced pathway for this IFN-mediated cellular turnover that could explain the enhanced functional proliferation seen in the ilea of KO mice.

In light of a recent paper suggesting that BrdU-labeled liver tissues could be used alongside unlabeled tissues for qRT-PCR analysis (12), the transcription levels of Irgm1 and β-actin were compared between BrdU-treated and untreated tissues. If our data were to confirm the suitability of BrdU-labeled tissues for RNA isolation and gene amplification, the number of mice needed for this and similar experiments would be cut in half. During my time in the lab, I was able to witness mouse breeding and handling and to be present at their daily weigh-ins and for several instances of mouse sacrifices and necropsies. Observing the tremendous amount of work that must be devoted to even a single experiment only fueled my interest in testing the traditionally held thought that BrdU impacts qRT-PCR amplification. Unfortunately, all but one group trended (p<0.15) toward differences (unreliably up- or down-regulated) after the BrdU labeling. The post-TBI WT jejunal tissues even showed a significant difference from their BrdU-labeled counterparts. Thus, we cannot recommend decreasing mouse numbers by performing quantitative experiments on BrdU-labeled tissues. Webster et al. worked with a different tissue (liver) and different BrdU administration method (via water bottles), so follow-up studies investigating whether the mode of BrdU administration determines its interference with qRT-PCR may be interesting.

In the future, further investigation into the role of other CD risk alleles, such as NOD2 and ATG16L1, on stem cell function may provide further elucidation into mechanisms of optimizing epithelial turnover and mucosal
healing in CD patients. I ran a preliminary qRT-PCR to assess whether NOD2 expression is upregulated post-TBI on
the WT samples used above and found a significant upregulation in both the jejunum (1.7X) and the ileum (4X). It
also may be worthwhile to assess the interferon levels, both Type I and Type II, of our mice and perhaps to assess
the proposed Wnt-independent pathway identified by Sun et al. If we discover that our Irgm1 KO mice also have
constitutively high Type I Interferons, this knowledge about our model could impact future experiments and the
ultimate CD treatments toward which they are intended. While the data from my summer project suggest a role
for Irgm1 in ISC function and epithelial regeneration, much more remains to be clarified regarding how the
complex ISC milieu might be manipulated to benefit the remission of CD patients.

VI. Works Cited

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