

# Netrin-1 guides inflammatory cell migration to control mucosal immune responses during intestinal inflammation

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**Abbreviations:** Adora2b, A2B adenosine receptor; AOM, azoxymethane; cAMP, cyclic adenosine monophosphate; CD, Crohn disease; DCC, deleted in colorectal cancer; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; Ntn-1, netrin-1; UC, ulcerative colitis; UNC, uncoordinated

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**T**he intestinal epithelium is a dynamic barrier playing an active role in intestinal homeostasis and inflammation. Intestinal barrier function is dysregulated during inflammatory bowel disease (IBD), with epithelial cells playing a significant part in generating an inflammatory milieu through the release of signals that attract leukocytes to the intestinal lamina propria. However, it is increasingly appreciated that the intestinal epithelium mediates a counterbalancing response to drive resolution. Drawing analogies with neuronal development, where the balance of chemoattractive and chemorepellent signals is key to directed neuronal movement it has been postulated that such secreted cues play a role in leukocyte migration. Netrin-1 is one of the best-described neuronal guidance molecules, which has been shown to play a significant role in directed migration of leukocytes. Prior to our study the potential role of netrin-1 in IBD was poorly characterized. We defined netrin-1 as an intestinal epithelial-derived protein capable of limiting neutrophil recruitment to attenuate acute colitis. Our study highlights that the intestinal epithelium releases factors during acute inflammation that are responsible for fine-tuning the immune response. Exploration of these epithelial-mediated protective mechanisms will shed light on the complexity of the intestinal epithelial barrier in health and disease.

## Introduction

An epidemiological study published within the last year demonstrated that

IBD, including Crohn disease (CD) and ulcerative colitis (UC) is significantly increasing in both incidence and prevalence worldwide, with North America having one of the highest reported prevalence rates.<sup>1</sup> Substantial evidence supports the hypothesis that in a genetically susceptible host an inappropriate immune response to endogenous microbiota or self-antigen promotes a persistent and pathogenic inflammation in the intestinal mucosa leading to IBD.<sup>2</sup> Mucosal inflammation as experienced during IBD is characterized by disrupted intestinal epithelial barrier function and inappropriate leukocyte accumulation in the intestinal lamina propria.<sup>2</sup> Significant efforts are being made to understand the mechanisms of epithelial barrier and immunological dysfunction during IBD. Initially considered as a mere physical barrier to the intestinal luminal contents, the intestinal epithelial cell layer is now known to be a dynamic force that directs mucosal immune responses.<sup>3,4</sup> Much information exists on the role that intestinal epithelial cells play in driving recruitment of leukocytes to the inflamed intestine through release of chemoattractants (chemokines).<sup>5</sup> However, there is increasing evidence that intestinal epithelial cells can mediate a counterbalancing response to attenuate inflammation.<sup>3,6</sup> Hypoxia driven signaling pathways have been highlighted as one such epithelial-mediated protective response in the intestine.<sup>7-12</sup> Original studies hypothesized that soluble anti-inflammatory factors were released by hypoxic epithelial cells. One such factor identified by these studies was the neuronal guidance molecule,

netrin-1.<sup>13</sup> Netrin-1 is member of a class of secreted guidance molecules initially recognized as being essential for neuronal development.<sup>14</sup> Novel studies point to an exciting additional role for netrin-1 in attenuating inflammatory responses in peripheral organs.<sup>13,15-18</sup> We hypothesized the hypoxic intestinal epithelium was a source of netrin-1 which could have immunomodulatory effects in a model of IBD. Our investigations revealed that netrin-1 is a key mediator acting to balance the acute inflammatory response during intestinal inflammation as observed in IBD.<sup>12</sup>

### Netrin-1 during Intestinal Homeostasis and Inflammation: An Epithelial Response

It is now evident that netrin-1 is part of an evolutionary conserved integrated network of signaling proteins that act simultaneously as chemoattractant or chemorepellent signals to control neuronal migration and development.<sup>19</sup> Considering the parallels between axonal guidance and leukocyte migration an exciting body of work demonstrates that netrin-1 plays a guiding role to modulate functional immune responses.<sup>13,15-17,20</sup> During IBD coordinated leukocyte migration is a key feature of disease pathogenesis.<sup>5</sup> We therefore sought to investigate if netrin-1 might play a role in IBD.

In vitro and in vivo studies demonstrated that netrin-1 is an endogenous mediator released in the intestine to suppress inflammation during acute colitis.<sup>12</sup> Mice with homozygous deficiency of netrin-1 do not live beyond postnatal day 3 due to improper axonal development,<sup>14</sup> therefore we conducted our studies using mice heterozygous for netrin-1 expression (*Ntn-1<sup>+/-</sup>*).<sup>12</sup> Initial findings revealed that in a model of acute colitis *Ntn-1<sup>+/-</sup>* mice experienced significantly exacerbated inflammation characteristic of acute disease compared with wildtype controls, including inflammatory cell influx and histological tissue damage.<sup>12</sup> Netrin-1 expression was predominantly observed in intestinal epithelial cells and myenteric neural units of the colon.<sup>12</sup> Netrin-1 has been observed to play an important role

in the developing enteric nervous system, where it is expressed by the endoderm and outer gut mesenchyme in the fetal mouse gut.<sup>21,22</sup> In line with our observations differentiated enteric nerves have been shown to express and synthesize netrin-1.<sup>23</sup> There is evidence that the enteric nervous system is functionally altered during IBD and neuronal-derived molecules have been demonstrated to exert anti-inflammatory effects in models of IBD.<sup>24</sup> Our studies revealed no differences in enteric neuronal anatomy in netrin-1 deficient mice during acute colitis, while netrin-1 expression in the colonic neural units remained consistent throughout the timecourse of colitis.<sup>12</sup> To date there is no evidence that the enteric nervous system of netrin-1 deficient mice is functionally impaired. From our observations we conclude that neuronal derived netrin-1 was not the predominant source of netrin-1 during acute colitis. However, the possibility that neuronal derived netrin-1 is an immunomodulatory factor in the intestine remains to be fully examined.

In contrast, the colonic epithelium expresses a high level of netrin-1.<sup>12</sup> It has been established that intestinal epithelial cells rapidly respond to environmental cues in order to control microbial invasion, intestinal barrier function and inflammation of the mucosa. This involves secretion of chemokines, cytokines and other factors that are responsible for controlling deleterious local responses.<sup>3-5</sup> Our studies point to the release of netrin-1 by the intestinal epithelium as an endogenous protective response in acute colitis.<sup>12</sup> In vitro studies revealed that cytokines relevant to the pathogenesis of IBD significantly increased netrin-1 expression in intestinal epithelial cells and epithelial netrin-1 expression was enhanced during the course of acute colitis.<sup>12</sup> These findings are in line with observations in a study of IBD patients where netrin-1 expression in mucosal epithelial cells was observed to be dramatically increased above that of control patients.<sup>25</sup> This group proceeded to demonstrate that enforced netrin-1 expression in the intestinal epithelium decreased epithelial cell apoptosis and predisposed mice to the development of colonic tumors.<sup>26</sup> Increased epithelial cell apoptosis and dysregulated intestinal

epithelial barrier function are hallmarks of IBD, as well as key features of IBD models.<sup>27,28</sup> Treatments that prevent epithelial cell apoptosis have been demonstrated to protect mice from inflammation in acute colitis.<sup>29</sup> In our studies mice treated with recombinant netrin-1 were protected during the course of acute colitis.<sup>12</sup> Based on these findings, we considered the hypothesis that netrin-1 control of epithelial cell apoptosis was responsible for alterations in disease outcome in our model. Surprisingly, analysis revealed that despite demonstrating exacerbated inflammatory cell infiltrate and histological disease netrin-1 deficient mice did not have increased epithelial barrier dysfunction or epithelial cell apoptosis compared with wildtype controls during acute colitis.<sup>12</sup> Similarly, administration of netrin-1 did not result in improved intestinal epithelial barrier function in vitro or in vivo. Despite evidence from cancer models, our studies led us to conclude that during acute inflammation as experienced in IBD, netrin-1 did not play a significant role in regulating intestinal epithelial cell apoptosis or barrier dysfunction. The exact reasons for this may be due to experimental design, choice of model and the timeline of disease progression. Original studies by Mazelin et al. utilized genetic overexpression of netrin-1 in the intestinal epithelium of mice.<sup>26</sup> This approach demonstrated that persistent netrin-1 expression led to significant epithelial cell hyperplasia by 20 months. Crossing netrin-1 transgenic mice with the adenomatous polyposis coli (APC) mice, a common model of colorectal cancer, resulted in an increased frequency of high grade adenomas compared with controls. The authors concluded that chronic elevation of netrin-1 inhibited apoptotic signaling in the intestinal epithelium.<sup>26</sup> These studies were followed up in the context of IBD associated colorectal cancer using the azoxymethane (AOM) plus dextran sulfate sodium (DSS) model.<sup>25</sup> Using a method to block receptor binding of endogenous netrin-1 in AOM/DSS over a period of 10 weeks it was observed that blockade of netrin-1 signaling decreased the frequency of high grade adenomas.<sup>25</sup> Neither study investigated the effect of netrin-1 signaling on

epithelial cell apoptosis at acute timepoints. Both studies highlighted the need for chronic netrin-1 signaling to enhance epithelial cell survival and focused on timepoints of 10 or 20 weeks. Our study used a distinct model of direct epithelial injury that is associated with epithelial cell apoptosis and acute inflammation, namely DSS colitis. In addition, our animal studies were of considerably shorter duration to either cancer model used.<sup>25,26</sup> These differences in model and timeline may in part account for our differential findings. In addition, the Mehlen group focused on the role of the netrin-1 receptors, deleted in colorectal cancer receptor (DCC) and the uncoordinated receptors (UNC5A-D),<sup>25,26</sup> Both receptor groups have been identified to regulate cell survival upon netrin-1 binding.<sup>30</sup> As yet the exact role for these two receptor families in IBD is unknown. Our study identified an alternative netrin-1 receptor, the A2B adenosine receptor (Adora2b) as the key receptor for mediating netrin-1 functional responses during DSS colitis (discussed below). Netrin-1 signaling through the Adora2b in our model of acute colitis may be an alternative reason why distinct netrin-1 effects were observed in our study. We cannot discount the possibility that netrin-1 may play a role in intestinal epithelial cell survival in a more chronic model of IBD. Taken together, our observation that netrin-1 did not affect intestinal epithelial cell apoptosis in acute colitis led us to consider other functions attributed to netrin-1 in peripheral organs.

### **Leukocyte Reverse Migration Mediated by Netrin-1 during Acute Intestinal Inflammation**

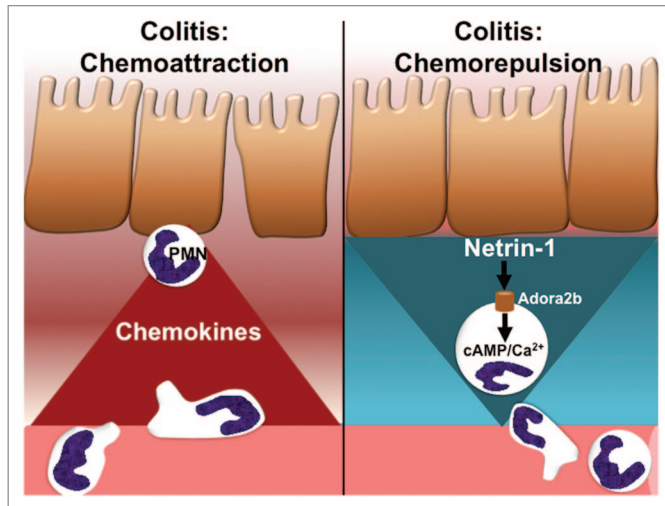
It is increasingly appreciated that leukocyte migration to sites of injury and inflammation is a complex phenomenon, regulated by a variety of signaling molecules and pathways.<sup>31</sup> Chemoattractants responsible for leukocyte recruitment are known to be expressed by intestinal epithelial cells during inflammation that occurs in IBD.<sup>5</sup> Much is known about chemokine-mediated leukocyte recruitment to the inflamed intestine, however the phenomenon of leukocyte egress from tissues is less well described.<sup>5,32</sup> The

existence of negative cues to reverse or prevent leukocyte recruitment are suggested by exciting studies performed in zebrafish, which demonstrate that neutrophils can retreat from sites of inflammation at the same rate that they are recruited to promote resolution of inflammation.<sup>33</sup> Due to similarities with the developing nervous system where neuronal guidance molecules supply both attractive and repulsive cues to steer appropriate neuronal patterning it was hypothesized that these molecules may have a role to play in regulating leukocyte recruitment during an active immune response. Initial investigations with this in mind demonstrated that netrin-1 was one such molecule that could inhibit leukocyte migration during acute inflammation.<sup>15</sup> Further studies in models of peripheral inflammation demonstrated that netrin-1 could inhibit neutrophil and macrophage migration to significantly reduce tissue inflammation and damage.<sup>13,16,17</sup>

During UC formation of neutrophil-filled crypt abscesses is a hallmark of disease.<sup>34,35</sup> Neutrophil infiltration into the colonic lamina propria is also a key feature of the murine colitis model used in our studies.<sup>36</sup> The definitive role of neutrophils during acute colitis is still debated. As discussed in a recent comprehensive review by Fournier and Parkos, neutrophils represent something of a “double-edged sword” in the context of intestinal inflammation.<sup>37</sup> During acute intestinal injury neutrophils play a vital protective role and can assist in the resolution of inflammation. However, inappropriate persistence of activated neutrophils at the site of inflammation can be detrimental during intestinal injury and IBD (reviewed in 37). Loss of a neutrophilic response was observed to result in a worse outcome of experimental colitis.<sup>38,39</sup> In contrast, suppressing neutrophil infiltration has been demonstrated to be an effective therapeutic intervention in a number of IBD models.<sup>12,40,41</sup> These dichotomous results suggest that complete blockade of a neutrophil response is likely detrimental, however suppressing an over-active response could be of benefit in IBD. Given the importance of neutrophil recruitment in colitis coupled with studies demonstrating an inhibitory effect

of netrin-1 on neutrophil and macrophage migration during acute inflammation prompted us to investigate the frequency of these cell types in our colitis studies. Detailed flow cytometric analysis demonstrated that netrin-1 deficient mice had a persistent and specific increase in neutrophil number within the colonic lamina propria during the course of colitis.<sup>12</sup> This observation could be reversed by netrin-1 treatment. Our analysis revealed no significant effect on monocyte or macrophage recruitment to the intestine indicating that netrin-1 exhibited preference for limiting neutrophil recruitment to the inflamed colon. Many signaling molecules have been identified that are involved in leukocyte recruitment to the intestine during inflammation observed in IBD (Fig. 1). We have identified netrin-1 as an endogenously expressed guidance molecule that can limit neutrophil accumulation to attenuate acute mucosal inflammation as observed in IBD (Fig. 1). Importantly, netrin-1 treatment does not result in complete blockade of the neutrophilic response, an approach which can be detrimental in this model (discussed above). We suggest that netrin-1 expression acts as a braking mechanism to suppress an over-zealous neutrophil response as observed in IBD.

Recent studies in atherosclerotic mouse models have pointed to a pathogenic role for netrin-1 guidance of macrophages.<sup>20</sup> This study implicated macrophage-derived netrin-1 as a retention signal for macrophages in the atherosclerotic plaque.<sup>20</sup> These differential findings to ours and other acute models point to a tissue specific and disease contextual role for netrin-1. Future studies would need to focus on a potential role for netrin-1 in the context of chronic intestinal inflammation, as IBD is a relapsing, remitting, long-term disease. Chronic inflammation observed in IBD and murine models is heavily reliant on lymphocytes,<sup>6,42</sup> therefore the tissue source and cell target for netrin-1 may be different in the context of chronic intestinal inflammation to that observed during acute colitis. The deleterious role of leukocyte-derived netrin-1 in atherosclerosis indicates that netrin-1 may have a distinct function in chronic models of IBD to that of acute colitis.



**Figure 1.** Balance between chemoattraction and repulsion mediated by epithelial cells during intestinal inflammation. The chemokines known to regulate neutrophil (PMN) migration into the intestinal lamina propria during colitis are well established. Our findings in an acute murine model of colitis present the neuronal guidance molecule netrin-1 as a chemorepulsive factor released by intestinal epithelial cells that limits PMN recruitment to the inflamed intestine. Initial studies point to netrin-1 signaling through the A2B adenosine receptor (Adora2b) as the mechanism by which netrin-1 mediates its therapeutic effects. Adora2b signaling can induce intracellular signaling molecules that have been demonstrated to alter PMN migration. Our study reveals that the intestinal epithelium can also be a source of chemorepulsive cues during acute colitis.

### Netrin-1 Signaling Pathways in Acute Intestinal Inflammation

An important aspect of netrin-1 function is the signaling cascade that it can induce. Netrin-1 can bind to a number of cell surface receptors, including the deleted in colorectal cancer receptor (DCC), the uncoordinated receptors (UNC5A-D), neogenin and the A2B adenosine receptor (Adora2b).<sup>43</sup> Signaling via the majority of the proposed netrin-1 receptors has yet to be investigated in IBD. Of note the Adora2b has been demonstrated to be a protective signaling pathway during acute tissue inflammation,<sup>44-49</sup> including colitis.<sup>50</sup> Considering our investigations demonstrated that netrin-1 mediated protection was driven by inhibition of neutrophil recruitment we proceeded to investigate netrin-1 signaling through receptors known to be expressed on this cell type. Previous findings have demonstrated that UNC5b is expressed on multiple leukocyte populations and have implicated netrin-1 signaling through this receptor in inhibition of granulocyte and lymphocyte migration.<sup>15,20</sup> Similarly, studies have demonstrated

that the Adora2b is expressed on neutrophils and signaling through this receptor inhibits neutrophil migration *in vitro* and *in vivo*.<sup>13,44,51,52</sup> Following a previously described protocol<sup>53</sup> we utilized antibody inhibition of the UNC5b receptor in our model of colitis. This proved unsuccessful in altering the protective function of netrin-1 during disease. In contrast, mice deficient in Adora2b could not be rescued from colitis progression by netrin-1 treatment, thereby implicating this receptor in netrin-1 mediated repression of neutrophil recruitment during acute intestinal inflammation.<sup>12</sup> Since its identification as a netrin-1 receptor the exact signaling mechanism by which Adora2b functions as a netrin-1 receptor has yet to be defined.<sup>54</sup> It is intriguing that studies link Adora2b signaling with both induction of cyclic adenosine monophosphate (cAMP) and also calcium mobilization.<sup>55</sup> The balance between these two intracellular signaling molecules has been implicated in determining directional migration in response to cues such as netrin-1.<sup>31</sup> We would predict that netrin-1 induces intracellular signaling pathways downstream of the Adora2b to inhibit neutrophil

migration. Future studies will seek to identify the specific molecular signature induced by netrin-1 to regulate reverse leukocyte migration during IBD.

### Conclusion

As our knowledge of the intestinal epithelial barrier increases it has become evident that intestinal epithelial cells are capable of active involvement in mucosal immune regulation. Analogous to the field of neuronal development it is tempting to suggest that intestinal epithelial secretion of guidance cues such as netrin-1 plays a fundamental role in directing the local immune response. Increasing our knowledge of the potential expression and function of these cues in the intestine may open up avenues of potential therapeutic intervention in IBD. It could be speculated that tipping the balance of mediators within the intestine toward a chemorepellent signature may be beneficial in reducing pathogenic leukocyte recruitment in IBD. We conclude from our studies that netrin-1 is a novel endogenous response generated within the intestinal epithelium to dampen neutrophil recruitment in acute colitis (Fig. 1). We would postulate that netrin-1 may have a beneficial function in chronic models of intestinal inflammation. However, recent findings in models of atherosclerosis identify leukocytes themselves as a source of chemorepellent cues to mediate leukocyte tissue retention. Therefore, future studies in IBD would need to consider the tissue source and dynamic expression of these guidance cues during the timecourse of disease to identify appropriate modalities of therapeutic intervention.

### Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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