THE CX3CR1+ LAMINA PROPRIA DENDRITIC CELL: MASTER SENTINEL OF THE TERMINAL ILEUM

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By

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ABSTRACT

The CX3CR1⁺ lamina propria dendritic cell (LPDC) of the terminal ileum provides a unique sentinel role, critical for intestinal health. Here, we demonstrate that three Crohn’s disease associated single nucleotide polymorphisms within the muramyl dipeptide receptor (MDP), NOD2, appear to alter the physical phenotype and transcriptome of the LPDC within the small intestine. Among the alterations described here, is a LPDC that is unable to extend its defining trans-epithelial dendrites (TEDs) into the lumen of the bowel despite (1) increased expression of the CX3CR1 receptor, (2) the presence of increased bacteria adherent to the mucosal wall and (3) the increased expression of the stress fractalkine, CX3CL1, all of which are directly associated with TED extension. In addition, the mRNA and protein expression profiles of the LPDC are also significantly distorted, including under-expression of genes within the TLR, ICAM and MIF families. There is also over expression of some inflammatory cytokines, such as IFN-γ and TNF-α, when comparing NOD2 mutants to wild types. Finally, the NOD2 mutation within the CX3CR1⁺ LPDC appears to depress Wnt5a expression both in vivo and in response to ex vivo MDP stimulation. In vivo observation of the Wnt5a/disheveled-2 axis showed reduced activation within the epithelium when in communication with the NOD2 mutant LPDC. However, once removed from the lamina propria compartment, the epithelium responded to
Wnt5a stimulation by augmenting the transcription of Tcf4, a Paneth cell dependent transcription factor involved directly in the expression of human alpha defensin 5. We propose that Wnt5a participates in a novel homeostatic circuit involving the LPDC, epithelium, and Paneth cell and that a mutation in the NOD2 receptor within this LPDC alters its ability to communicate with its immunologic epithelial counterparts allowing for bacterial infiltration of the mucosal niche.
The research and writing of this thesis is dedicated to everyone who helped along the.

Many thanks,
Denver M. Lough
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## Examination of a Circuit: Primary Research on the NOD2 Defect in the CX3CR1+ LPDC

*Mutations in the Human NOD2 Gene are Associated with an Altered CX3CR1+ Intestinal Lamina Propria Dendritic Cell*

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INTRODUCTION

A BRIEF INTRODUCTION TO CROHN’S DISEASE

The small intestine, in particular the terminal ileum, contains the largest lymphoid tissue aggregate within the human body (1). This highly developed lymphatic region, located proximal to the ileo-cecal junction, is a site that is commonly affected during Crohn’s disease (CD) (Figure 1). The correlation between this anatomical setting and the intense inflammatory symptoms developed within CD are thought to develop following the breakdown of a homeostatic relationship that exists between “self-tissues” and the microbiome that is present in such close contact within the bowel lumen. This loss of homeostasis is subsequent to a figurative “tipping point” where disequilibrium between microorganisms that normally populate the lumen, are able to proliferate to numbers far greater than “normal” basal levels and eventually overtake the mucosal barrier (2).

This imbalance, or “tipping point,” that occurs is believed to be interdependently coupled within an abnormal immuno-modulatory circuit that feeds off the dysregulation between both the innate and adaptive arms of the immune system which compounds local tissue destruction. Beyond improper augmentation of the inflammatory processes between the two arms

Figure 1: Inflammatory location of CD. Image taken from: http://en.wikipedia.org/wiki/File:Patterns_of_CD.svg
of the immune system, one can also see mis-communication between the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5+) stem cell-derived epithelial compartment of the small intestine and the juxtaposed lamina propria compartment, which contains bone marrow-derived immune cells capable of mounting an intense inflammatory response often indiscrimate of the target (2) (3) (4). This interface is essentially the keystone between barrier protection, the epithelium, and the effector immune cells that populate the lamina propria. Here, at this interface, an enormous amount of information can be learned with regards to mechanisms in gut inflammation.

With so many factors concerning the patho-biology of CD being studied currently, one could easily say that the cause is multi-factorial. Moreover, the intrinsic architecture of the small intestine, in that it possesses these separate yet integrated compartments (epithelial vs. lamina propria), results in a circular “cause-and-effect” pathway that can easily become over stimulated, as it senses feedback directed pro-inflammatory signals. At some point, this overstimulation leads to the destruction of the delicate intestinal harmony which exists between self-tissues and microbes, ultimately developing into a milieu of disruptive inflammatory factors. This disruption has been given the “blanket term” of “chronic inflammatory bowel disease” since no one factor has been designated as the causative variable within the process. With
such chaos occurring at the epithelial wall (Figure 2.), it comes as no surprise that the etiology of CD is often referred to as “unknown (1).”

Figure 2 describes the classic hypothesized inflammatory circuit that exists among microorganisms, the intestinal epithelium and the bone marrow-derived immune cells that populate the lamina propria compartment. This figure depicts a bacterial insult to the wall of the intestine and a misdirected response by the bone marrow-derived immune cells (antigen presenting cells and and T-cells) in that they are oriented more toward an intracellular or cytotoxic cellular response, rather than the typical extracellular focus commonly seen against bacteria. This deviation from the “normal” response is generally what is thought of as a defining cellular characteristic among CD.

In addition, Figure 2 also subtlety depicts the complexity of variables and research topics found within the literature to date. Depending on one’s focus, many arguments can me made about the initial “spark” that begins the process in patients with inflammatory bowel disease. Those who are microbiologist or more “bacterio-centric” will argue that there is a pioneer organism, similar to the ability of H. pylori in the initial development of gastric ulcers, which penetrates the mucosal barrier in order to weaken epithelial defenses. This organism may lay dormant for years before gaining enough destructive momentum to offset native human mucosal fortifications. Other’s who would consider themselves to have more of the molecular biologist mind frame will argue the instigating spark relates to improper anti-microbial peptide processing, resulting in a dysfunctional of possibly malformed protein and a subsequent inability to kill bacteria. While someone who may consider themself more of a cellular biologist or immunologist would suggest that the immune cells that populate the lamina propria are providing

3
inadequate signals to the micro-environment and therefore hierarchically dictate the state of small bowel’s mucosal niche incorrectly. In other words, the genetic profile of the immune cells, which are initially developed in the bone marrow, determines how these cells will act once in the small bowel environment. Moreover, these cells are trusted with the proper regulation of the inflammatory response necessary to monitor and respond to the ever-changing luminal contents, including microorganisms. Dysfunction in these immune cells, not only incapacitates these cells in dealing with bacteria, but places the mucosa in the crossfire and at risk of becoming a casualty of the maladapted immune response.

What will be learned throughout this brief review or survey of the literature is that there is no single answer to the pathology of Crohn’s disease and that all theories relating to the cause, have significant value in developing a possible cure in the future. Furthermore, this review will shed light on how the protection of the gut wall employs a complex systematic circuit which requires multiple key players (cellular, molecular and immunologic) in order to respond properly to the continuous challenge that microorganisms pose when in such close proximity to delicate intestinal tissue.

**A BRIEF HISTORY OF THE CLINICAL RELEVANCE OF CROHN’S DISEASE**

With more of a relevant clinical definition than a true basic science mechanistic description, Crohn’s disease remains one of the most common intestinal diseases in man (5). Named after the American gastroenterologist Burrill Bernard Crohn, who, in 1932, discovered and documented the disease as a type of “regional ileitis,” CD has since continued to plague western developed societies with little advancement toward a cure
outside basic pharmacological immune suppression and end-stage intestinal transplantation (6). Until now, CD has been defined as a clinically recognized chronic inflammatory disease state of the intestine, which must meet certain criteria in order to adopt the diagnosis. In addition to the underlying chronic effects, CD patients undergo severe and subsequently worsening life-threatening flare-ups, which often lead to hospitalization, scarring of the intestine and eventually death.

CD, placed under the umbrella term of inflammatory bowel disease (IBD) along with Ulcerative Colitis (UC), another chronic inflammatory disease of the intestine, has conventionally been described as a multi-factorial disorder in that its patho-biology is associated with both genetic and environmental factors which alter the “normal” host/bacterial interaction that occurs at the mucosal membrane within the GI tract (4) (7). The deviation from “normal” commensal interactions eventually leads to an altered inflammatory response state where the production of inflammatory mediators (chemokines, cytokines, interleukins) are often up regulated and expressed in abnormal ratios when compared to the normal basal un-inflamed intestine (8). This skewing toward a more inflamed state leads to greater tissue destruction and consequently more invasion of the gut tissue by bacterial organisms, further feeding the cycle.

It was not until this last decade, that researchers knew much about genetic or molecular associated markers for CD. Recently, using RT-PCR and genomic sequencing, single nucleotide polymorphisms (SNPs) among the nuclear oligomerization binding domain-2/caspase recruitment domain family-15 gene (NOD2/CARD15) were documented to be associated with an increase susceptibility to Crohn’s Disease (9). Three of these SNPs within the gene, were shown to lead to a forty fold increased relative risk
ratio among those people who contain one or more of these mutations within their genome. Although there are more than fifty identified SNPs within the NOD2 allele that have been discovered in humans, it is only these three that have significant associations with the development of CD. Some specialized medical centers, including Georgetown University Medical Center’s Transplant Institute, genotype patients with clinically diagnosed CD in order to better understand the NOD2 association with the disease phenotype. (Table 1.) (9).

**Table 1. Crohn’s-Associated Single Nucleotide Polymorphisms of NOD2 Allele.** Three of the most common single nucleotide polymorphisms present in the NOD2 gene leading to increased risk of developing Crohn’s Disease. Data acquired using NCBI Entrez Gene database. Description of the mutation and reference identifiers is included.

<table>
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<th>Type of Mutation</th>
<th>Genomic Alteration</th>
<th>Protein Alteration</th>
<th>NCBI SNP ID</th>
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<td>(1)</td>
<td>a missense mutation</td>
<td>2104 C→T</td>
<td>SNP 8; R702W</td>
<td>rs2066844</td>
</tr>
<tr>
<td>(2)</td>
<td>a missense mutation</td>
<td>2722 G→C</td>
<td>SNP 12; G908R</td>
<td>rs2066845</td>
</tr>
<tr>
<td>(3)</td>
<td>a frameshift C-mutation</td>
<td>3020insC</td>
<td>SNP 13; 1007fs</td>
<td>rs2066847</td>
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With roughly 1-3% of the general population carrying one these three SNPs it comes as no surprise that CD is one of the most common gut pathologies in man. Interestingly however, there are approximately 40% of CD patients who carry one or more of these SNPs in the NOD2 allele. This means that although a mutation in the NOD2 allele is a strong predictor of developing CD, it is not the sole indicator, and that other variables seem to co-exist with the development of the disease. With such non-
definitive genomic data, there is still a need to understand the causative molecular mechanism(s) of the disease so that we can one day begin to orient ourselves toward a cure rather than simply treating the symptomatology of the classic disorder.

A model that begins to shed light on this complex multi-cellular circuitry of CD has recently been found in the small intestinal transplant population. This human allograft model is unique to the study of gut inflammation and/or CD, in that it provides researchers with chimeric intestinal tissues where donor and recipient cells can interact at a seemingly dynamic interface. Here, the chimera is developed over time, within the intestine, between two compartments of the gut wall. These separate compartments are defined as:

(1) The epithelial compartment

(2) The lamina propria compartment

These two separate, yet interdependent compartments, remain genetically donor and recipient in origin, respectively, throughout the life of the organ. The epithelium itself persists as donor derived, in that the bowel that is ex-planted from the donor maintains the ability to reproduce due to the epithelial-based Lgr5+ stem cell which persists deep within the crypts of the mucosal epithelium. The lamina propria compartment, while initially donor in origin, in that it harbors donor bone marrow-derived immune cells, undergoes a gradual repopulation event. This repopulation of the lamina propria compartment occurs slowly over time while the recipient populates the allograft with their own bone marrow-derived immune cells and donor cells apoptose or are lost in the lumen. It is this migration of the bone marrow-derived immune cells, bringing their genotype and resultant phenotype into the intestinal niche, that allows the
transplant to serve as a model for the NOD2 variable within specific cell types. For example, a NOD2 mutant recipient patient receiving a NOD2 wild type intestinal allograft, will theoretically develop a chimeric bowel at some point after the vascular anastomoses, resulting in a lamina propria compartment containing NOD2 mutant immune cells and an epithelium, which remains NOD2 wild type.

What is curious about the repopulation dynamics leading to true chimerism within the gut is that among those patients who undergo intestinal transplant, 40% of recipients carry one of the three primary CD-associated NOD2 SNPs. Having one of these three SNPs within the bone marrow genome is highly correlated with poor outcome (higher infection rate, rejection rate and loss of graft) in addition to increased mortality in the patient population (10). These findings suggest that the bone marrow-derived immune cells, those which carry NOD2 mutations, in some way re-orient the innate immune state of the bowel epithelium toward a Crohn’s reversion event.

This allograft model, where the donor intestine carries a wild type NOD2 allele and the recipient bone marrow carries a NOD2 mutant SNP, appears to uncover the possible pathological circuitry concerning an initiating event among Crohn’s disease patients. This prototype puts more emphasis on NOD2 function within the immune cells rather than among the cells that construct the epithelium of the gut. This becomes apparent as the NOD2 mutant bone marrow-derived immune cells begin to repopulate the NOD2 wild type graft and associated symptoms become evident within the patient and the bowel. Within murine models, particularly among NOD2 knockout and siRNA knockdown mice, researchers have shown to that they are able to reduce the expression of the NOD2 gene within whole tissues and certain cell types in order to prove an
association between the NOD2 defect and bacterial accumulation. However, the human intestinal transplant model itself uniquely permits the bone marrow reservoir of immune cells to slowly repopulate the graft, allowing researchers to track the onset of inflammation and bacterial invasion as it relates to the relative percentage of recipient chimerism among bone marrow-derived immune cells. In a case where the recipient carries a NOD2 SNP, one could begin to associate the percentage of defective cells required to develop the onset of pathology or a fixed pathological outcome. The limits of murine models become more apparent when discussing the significant differences that exist within the epithelial production of anti-microbial peptides and gut bacterial species. Although there is published literature on the NOD2 KO mouse, it suggests only “what can happen” within the human gut rather than “what does happen” when a NOD2 wild type and NOD2 mutant interface is developed.

**A BRIEF INTRODUCTION TO THE NOD2 GENE IN CROHN’S DISEASE**

The NOD2 gene, also known as the inflammatory bowel disease-1 gene (IBD-1gene), remains the strongest predictive genetic marker for the development of Crohn’s Disease (9) (11). In addition to NOD2, two other genes, a fractalkine receptor called CX3CR1 and an autophagy pathway gene, ATG16, have become topics of interest within the CD research arena (12). However, these genes still require more research and high throughput genetic evaluation to truly determine a significant associative role. At this time, NOD2 is the sole genetic-associated predictor for the development of CD on the NCBI database, Online Mendelian Inheritance in Man (OMIM). This is certain to change in the near future as more genetic prevalence studies surface internationally.
With NOD2 serving as the pioneer CD gene, one must begin to understands its role and function within cells. The association between NOD2 function, mutation and pathologic outcome makes sense if one begins looking at the protein form encoded by the NOD2 gene itself. Simplistically, NOD2 is thought of as a three-part protein, which contains an intra-cytoplasmic pattern recognition receptor (PRR) capable of sensing bacteria (13). This three-part structure of the functional receptor (1040 aa.) contains two caspase recruitment domains, termed CARDs, which are both located at the amino terminus of the peptide. CARD1 and CARD2 are followed downstream by a nucleotide-binding domain or NBD. Moving down the coding region, toward the carboxyl terminus, are ten leucine-rich repeats or LRRs (11). These three regions of the gene encode pertinent functional structures, pivotal to the role of NOD2 as a microbial sensor. For example, the LRR region of the receptor identifies and interacts with bacterial muramyl-dipeptide (MDP), while the CARD regions interactively communicate with the downstream effector molecules within the immune response pathway (14).

**HOW EACH REGION HELPS TO DEFINE THE FUNCTION OF THE NOD2 GENE**

In order to better define the function of NOD2 based on its structure, each region should be understood in detail. Following this, one can see how alterations in the function of the gene/protein relate to the development of CD. Furthermore, it may orient the defective mechanism toward an hypothesis involving the bone marrow-derived antigen-presenting cell within the lamina propria. The LRR region of the NOD2 gene, described in 1985, as a repeating 24 amino acid sequence, contains many hydrophobic residues. This hydrophobic sequence has now been shown experimentally to recognize a
bacterially derived glyco-peptide. This glyco-peptide, known as muramyl dipeptide or MDP, possesses a specific antigenic structure:

$$\text{MurNAc-L-Ala-D-isoGln,}$$

and is a component of the cell wall of virtually all bacteria (15). The sequential repetitiveness of the LRR region forms what appears to be an “arc-like” structure depicting a “U” with respect to the development of the section’s 3-dimensional orientation. This region has been commonly referred to as a “horseshoe,” within the literature, due to the orientation of hydrophobic amino acids resolving themselves inward and thus pointing toward the core region of the concavity. Here, within this architectural core, is a location where the ligand (MDP) binding occurs, defining the NOD2 receptor as a true pattern recognition receptor (16).

Following interaction with its ligand, MDP, the NOD2 protein undergoes an induced conformational change that allows it to interact with a downstream adaptor molecule known as receptor interacting caspase-like kinase (RIP2). This interaction occurs through the amino terminal CARD1 and CARD2 domains. It is here, that the CARD domains, which are found in a number of pro-apoptotic proteins, such as caspase-1 and caspase-9, function as adapters which mediate the activation of a pro-inflammatory pathway cascade. Experimentally, it has been observed that CARD activation of RIP2, within this NOD2 activation pathway, subsequently leads to the induction of poly-ubiquitination of IκκB, a protein which acts as both a scaffold and molecular modulator of NF-κB. Following the removal of IκκB, via this ubiquitin-proteosomal destruction pathway, NF-κB is free to alter the expression of an arsenal of inflammatory cytokines (Figure 3.) (17).
Beyond the basic structure-function relationship of this protein, the NOD2 multidomain receptor is expressed mainly by antigen-presenting cells such as myeloid-derived monocytes, macrophages and the dendritic cell (18). In addition, other studies have detected the NOD2 protein within the enterocyte and Paneth cell of the small intestine; although, its ability to truly function within these non-APC cell types remains to be determined (19) (20).

![Diagram of the NOD2 receptor protein upon stimulation with MDP as described in above text.](image)

**Figure 3.** Diagram of the NOD2 receptor protein upon stimulation with MDP as described in above text. The sequence of events is as follows: The hydrophobic core horseshoe of the LRR region binds MDP and changes conformation to allow CARD1 and CARD2 to bind RIP2. This interaction with RIP2 permits the poly-ubiquitination of IkB subsequently releasing NF-kB which then activates downstream inflammatory effector transcription factors.

The role(s) of NOD2 among the myeloid derived APCs that exist within the submucosa and lamina propria of the small intestines have become clearer since studies evaluating alterations in the function of the gene have been linked with changes in
downstream pro-inflammatory effectors. Recently, it has been shown that individuals with CD-associated NOD2 SNPs over-express several of the NF-κB target genes. These genes include many of the pro-inflammatory molecules such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and some members of the interleukin family such as IL-6 and IL-1β (21) (22) (23).

Interestingly, researchers have discovered that when conducting experiments where APCs, after transfection with the NOD2 SNP, were no longer capable of activating the NF-κB pathway when these cells were challenged with the NOD2 ligand, MDP (24). This again adds uncertainty to the true pathobiological mechanisms involved in augmenting an immuno-modulatory responsive element following microbial stimulation. Since the findings of multiple studies suggest dichotomous results with regards to NF-κB pathway expression regulation, the field is left with the conclusion that there is no single cause but rather a barrage of variables involved in this chronic inflammatory disorder surrounding a relatively complex circuit, which certainly involves more than one step in the NF-κB pathway.

While, many members of the scientific community stake claim to a single or more important element in the development of CD, it becomes clear that after a broad review of the literature, several of the concepts and/or mechanisms involved in the precipitation of this pathology appear to present strong evidence that there may in fact be a multivariate web of interdependent events that present the disease from different angles depending on the initiating event. These arguments, however, cannot disregard the fact that there may be a feedback circuit that might involve multiple cell types and proteins that are theoretically all important in gut homeostasis.
From this understanding, what is apparent when evaluating the molecular biology of NOD2 is that the SNPs associated with the development of Crohn’s Disease-like symptoms appear to modify the domain or structure of the leucine-rich-repeat region of the receptor. This fact alone, provides strong evidence for a role in the increase in biomass above what is considered to be a physiologic level of luminal microorganisms. Moreover, since it is understood that the interaction of the NOD2 receptor with bacteria occurs at the LRR domain, and that this binding permits a ligand-induced conformational change to the receptor leading to the CARD binding of RIP2, it is plausible to suggest this structural alteration could lead to downstream effects that ultimately dysregulate the NF-κB pathway. This would suggest that a SNP mutation within the LRR would not only fundamentally prevent the receptor from doing its primary job of conveying bacterial levels to the cell, but also alter all downstream events regulated by the CARD→RIP2→NF-κB pathway, namely pro-inflammatory/anti-apoptotic processes (9).

This strong association between the defect in the NOD2 gene and the development of Crohn’s disease is particularly interesting and should continue to be studied. However, merely associating SNPs with a disease and the site-specific alteration is not enough to generate a potential cure, particularly when the details surrounding the NF-κB pathway are still not well understood. Alterations in the gene have been shown to change the NOD2 binding dynamics of the protein to RIP2; however, few studies have looked at how compound SNPs or homozygous mutations alter behavior of the protein behavior beyond the NF-κB pathway.
INTRODUCING A SENTINEL ANTIGEN-PRESENTING CELL ARMED WITH NOD2

It is this myriad of complex variables concerning homeostasis among bacteria, epithelium, immune cells and inflammatory pathways which begins to shed light on the true intricacy of this topic involving microbial sensing, processing and finally effector pathway integration within this intestinal immuno-modulatory circuit. Being able to acquire and integrate so many complicated signals requires a special sentinel cell, able to communicate with other cell types within its surroundings. It is this region, or intestinal niche, that requires such a sentinel cell to develop complex and integrated communicatory signals. These signals must be powerful, yet focused, in order to alter the micro-environment enough to fend off the billions of microbes that pass over the mucosa daily. To define it bluntly, approximately one-third of total fecal weight is bacteria in nature. It is within this potentially infectious environment that the recently discovered CX3CR1+ lamina propria dendritic cell possesses a unique spectrum of qualities that permit it to migrate from the bone marrow, through the blood, to home regions of the terminal ileum which come in contact with microorganisms.

This CX3CR1+ LPDC, once properly oriented within the lamina propria compartment of the mucosa, travels to a region just below the basal lamina of the epithelium and extend a unique structural process across the sophisticated epithelial barrier into the lumen. These processes, termed trans-epithelial dendrites (TEDs), act via an enterocyte-derived CX3CL1 ligand-induction system, in which the CX3CR1 protein receptor on the LPDC is specifically advanced through the tight junction anchoring proteins, allowing access to the intestinal lumen safely without compromise to the mucosal integrity (25). Upon entrance of the TED into the mucin layer overlying the
mucosa, a vast array of extracellular and intra-cytoplasmic microbial pattern receptors are used to detect potential commensal and/or pathogenic organisms that come too close to the host’s epithelial barrier (26) (27) (28). One of these pattern recognition receptors is none other than the intra-cytoplasmic MDP-binding protein NOD2.

It is after this sensing occurs that the CX3CR1+ LPDC integrates the pattern’s information profile about the potential threat and develops an effector response, which is communicated to the surrounding cells via cytokines and other inflammatory ligands. With such abilities, the CX3CR1+ LPDC of the lamina propria compartment plays a pivotal sentinel role in maintaining the homeostasis of the terminal gut. Without this cell, it is almost certain that a chaotic state would ensue within the intestine following challenges by external stressors.

Here, the concept of a sentinel cell, capable of using the PRR, NOD2, at the front line—directly at the mucosal wall—begins to shed light on how NOD2 mutations can improperly alter the message sent by the informant LPDC to the local epithelial microenvironment. Preventing proper bacterial recognition through basic evolutionarily conserved patterns essentially “blinds” the intestine from knowing how many and what kind of organisms are within close proximity and consequently could become a threat. Is there LPS, or CpG islands, MDP, flagellin etc? These bacterial components can be recognized through the LPDC pattern recognition receptors.

At this time, a movement toward understanding this cell’s role in the NOD2 circuit of intestinal immunology appears to be a more causative or hierarchical approach by CD researchers. Past studies have typically focused on a sole cytokine, anti-microbial peptide or bacterial species as being a sign of intestinal unrest, rather than possibly
NOD2 causing these alterations congruently or in parallel with each other. Defining the role of the LPDC and its vast web of immuno-modulatory communications within the micro-environment and among other immune cells could certainly provide the putative cause of CD based on a more well defined NOD2 defect causing or initiating the malfunctioning circuitry.

THE IMPORTANCE OF THE EPITHELIAL COMPARTMENT: SETTING ITS ROLE IN CD

Beyond the bone marrow-derived antigen-presenting cells that were previously introduced, the epithelium is also a major player within the innate immunity arm of the bowel, particularly at the terminal ileum. To start, the epithelium possesses the capacity for high turnover, which prevents microbial adherence via a somewhat dynamic and mechanical process, known as desquamation. If the cells, namely the enterocytes, are constantly moving up the crypt and the villi, materials and organisms are much less likely to adhere and accumulate for any given amount of time. If materials or bacteria do, for some reason, come in contact with the epithelium and manage to attach to a cell, the enterocyte lifespan is very short (4-6 days), permitting the cell to detach and die off in the lumen of the gut.

Along with the capacity of high turnover, the epithelium also has the ability to produce anti-microbial peptides (defensins, lysozyme, phospholipase-A2, etc.). These compounds chemically diminish the bacterial load along the mucosa and within the mucus layer by altering the bacterial external structure and punching holes in their cell walls and membranes. These membrane and cell wall alterations kill the organism through disequilibrium or inversion of their electro-chemical gradient. In short, the
intestinal epithelium is a somewhat formidable opponent to the $10^{14}$ microbes that populate the bowel. Although there are more bacterial cells on and within the human body than there are human cells, the intestine remains a difficult environment for microbes to manipulate and overcome within a healthy individual (29). These unique properties of the intestinal epithelium provide the mucosa with an invaluable barrier system capable of interacting with the CX3CR1$^+$ LPDC in a joint effort to maintain commensal microbiota and evade potential pathogens (30).

Appreciating this highly intricate and interdependent association between the bone marrow-derived CX3CR1$^+$ LPDC of the lamina propria compartment and the cells which make up the epithelial compartment requires some fundamental knowledge of the biology of the bowel epithelium. First and foremost, the intestinal epithelium is the most rapidly self-renewing tissue in the adult mammal, making the intestinal stem cell, found deep within the crypt, a highly active cell within this region. This highly active cell is defined by a leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5$^+$), giving it the name “Lgr5$^+$ intestinal stem cell” or “Lgr5$^+$ ISC.” It is unique in that it is capable of inducing rapid alteration in the mucosal profile via proliferation of multiple epithelial progenitors into functional and responsive cell domains located along the crypt-villus axis (31).

The basic micro-physiological structure of the small intestine, termed the crypt-villus axis, has since been shown to be a self-organizing unit developed from a single Lgr5$^+$ intestinal stem cell (ISC) (32) (33). From this stem cell, 5 known separate cell types can develop which are depicted below (Figure 4.)
Figure 4. The anatomy of the small intestinal epithelium crypt-villus axis. The epithelium is shaped into crypts and villi (left). The lineage scheme (right) depicts the stem cell, the transit-amplifying cells, and the two differentiated branches. The right branch constitutes the enterocyte lineage; the left is the secretory lineage. Relative positions along the crypt-villus axis correspond to the schematic graph of the crypt in the center. *Figure in its entirety Radtke F, Science 2005*

(1) The columnar enterocyte (making up 95% of epithelial cells and performing the vast majority of nutrient absorptive function.

(2) The goblet or mucous cell

(3) The entero-endocrine cell

(4) The Paneth cell, which produces the human alpha defensins 5 and 6
The M-cell (membranous or micro-fold cell) which is found close to the Peyers patch and assists in antigen transport (34).

The ability to derive lineage-specific cell types from the Lgr5+ stem cell, permits self-organization of the crypt within spatially distinct compartments and ultimately adds to the stem cell’s unique role and capacity to respond to the ever-changing challenges that exist within the bowel.

**THE LAMINA PROPRIA COMPARTMENT: A HOME TO THE CX3CR1 LPDC**

Early in 2005 a myeloid-derived dendritic cell that populated the lamina propria of the terminal ileum was discovered and identified in the mouse. This dendritic cell was termed the CX3CR1+ lamina propria dendritic cell, or CX3CR1+ LPDC. A key sentinel of the intestine, the CX3CR1+ LPDC, is capable of maintaining the homeostasis of the intestinal mucosa by essentially “tasting” bacteria and directing an immuno-modulatory effect through both innate and acquired immune response system pathways (26) (30) (35) (36) (37). The CX3CR1 trans-membrane protein, for which the LPDC is named, mediates the dendritic cell’s access to the intestinal lumen, permitting this essential bacterial clearance response mechanism (25) (37). Not only has this cell been shown to permit the clearance of bacteria through a CX3CR1-mediated process, but it was also found that when the CX3CR1 knockout mice were challenged with a bolus of *Shigella typhimurium*, the defining trans-epithelial dendrites (TEDs) did not extend (Figure 5). This lack of TED extension, led to greater bacterial accumulation on the mucosal wall, eventually
Figure 5. CX3CR1+ LPDC in mouse ileum. (A and B) Depict the normal trans-epithelial dendrite extension which occurs with a functional CX3CR1+ LPDC under bacterial stimulation. (E and F) Depict the lack of TED extension with a correlating mutation in the CX3CR1 gene. The figure was designed to depict the idea that trans-epithelial dendrite extension requires the CX3CR1/CX3CL1 axis in order to extend the processes toward bacteria in the lumen of the small bowel. The partial figure was taken from (25).

permitting invasion by the microbes into the host tissue. The study also showed that without antibiotic intervention, mouse mortality significantly increased secondary to septicemia caused by the invading organisms. These published findings, not only suggest that the CX3CR1 protein is involved in TED extension at the mucosal/bacterial interface, but that CX3CR1 is directly required for the interaction to take place, and that without it, there is a breach of the epithelium by microorganisms since the sentinel cell is seemingly blind to their presence.
From this simple, yet informative and pivotal study, researchers determined that LPDC was found to regulate the clearance of entero-invasive organisms via the CX3CR1 protein within the intestine. However, this was not the first time an intestinal dendritic cell, located within the mucosa was thought to play a functionally substantial role in antigen processing as it related to gastrointestinal health. Researchers in 1996, described an intestinal Dendritic cells (DC) representing a major population of MHC-II "professional" antigen-presenting cells within the intestine. At that time, researchers thought that this professional MHC-II+ DC was distinct from cells of the monocyte/macrophage lineage, yet was capable of extending “pleomorphic processes,” which were able to establish a complex and organized communication network. This network was thought to regulate and augment intestinal antigen processing, as well as T-cell activation, in the context of allergic and infectious diseases in the gastrointestinal tract (38).

In addition to simply identifying this pleomorphic cell type and associating its activity with T-cell behavior, further observational studies were performed in order to more fully understand the ability of the DC’s to penetrate or “open” the interlocking tight junctions that exist between epithelial cells of the intestinal wall (37). It was understood that the extended DC process could not simply punch through the epithelial tight junctions, in that this action would create holes or breaches within the mucosal wall, leading to barrier compromise in a dangerous external environment. Instead, researchers suspected a more complex method of interaction between the LPDC and the enterocyte of the epithelium, where in the integrity of the barrier would be maintained.
Maintaining this competent intestinal-luminal barrier, while sending dendrites across the epithelium in order to directly sample the local microbiome of the gut, requires a more sophisticated method of access than simply poking holes through enterocytes. This mechanism, although directly dependent on CX3CR1 for TED extension, could not occur without the DC having the unique additional ability to delicately cross tight junctions. This curious phenomenon led researchers to discover another exceptional ability of the CX3CR1+ LPDC to express tight-junction proteins on the external surface of the TED. These tight junction proteins, such as occludin, claudin 1 and zonula occludens 1, permit the TED to work its way through the intricate connection while integrating its own self into the impermeable barrier (39). A more in-depth analysis regarding this unique ability will be discussed in the section pertaining to cytoskeletal alterations of the CX3CR1+ LPDC.

**A Brief Introduction to the CX3CR1/CX3CL1 Axis**

The fact that this particular fractalkine receptor/ligand protein pair is integral to the function of the LPDC begs the question as to what is known about it. CX3CR1 or chemokine (C-X3-C motif) receptor 1, as defined by NCBI’s ENTREZ GENE, has been defined as: V28; CCRL1; GPR13; CMKDR1; GPRV28; CMKBRL1; CX3CR1 in previous gene and protein curations, as per REF SEQ identifier NM_001337 (NCBI). The CX3CR1 gene has been recognized as a putative homolog within humans, chimpanzee, dog, cow, mouse, rat, and chicken according to HomoloGene 2035. This gene/protein product has also been implicated recently in the progression of HIV, in that the encoded protein is also a co-receptor for the virus. Interestingly, it has been discovered that of
some variations in this gene lead to increased susceptibility to HIV-1 infection and rapid progression to AIDS (40).

Up until 1997, little if anything was known about CX3CR1, and at that time, there were only three known major structural divisions or subfamilies within chemokine peptides. These three major forms were generally accepted as (Figure 6.):

1. The (CXC) form
2. The (CC) form
3. And the (C ) form

![Figure 6. The four main chemokine family structures.](image)

The three classic chemokine structures aligned on the top row of the diagram are C, CC and CXC respectively. CX3C is unique in that it possesses a mucin stalk which assists in the attachment of the chemokine via a GPI anchor to the basal side of an enterocyte membrane. Here, when bound, the CX3CL1 chemokine structure acts as adhesion peptide for the CX3CR1 receptor. The partial figure was taken from reference (41)

These structural terms correlate with the number of, and spacing between, the first two cysteines within the defined and conserved 7 pass trans-membrane structural motif
These motif variations, not only alter the apparent external structure of the protein itself, but also modify downstream function. These separate chemokine forms recruit and mobilize certain types of cells and further direct these specific cells to secrete a focused milieu of chemokines upon stimulation. For example, the (CXC) form or subfamily motif appear to interact more with polymorphonuclear cells (PMNs), also known a neutrophils, than other bone marrow-derived immune cells. However, the (CC) subfamily of chemokines appear to attract mainly monocytes as well as some lymphocytes, basophils, and eosinophils. Moreover, the last of the conventional chemokines, the (C ) forms, appears to act preferentially on lymphocytes (42) (43) (44). For decades, it was thought that these three motifs dictated the recruitment or attraction of their defined cellular subsets until recently a new subfamily was revealed.

It was upon the discovery of a fourth ligand, fractalkine or CX3CL1, that researchers realized that there was more variation than had been traditionally thought. This fourth chemokine, Fractalkine (FKN), also known as CX3CL1, is a unique membrane-bound molecule expressed on endothelial, epithelial and dendritic cells. The CX3CL1 ligand appears structurally similar to the CXC, CC and C forms, its CX3C motif alters not only its receptor interactions but also its function. The ligand CX3CL1 is constructed of both a chemokine domain and a rather long mucin-like stalk or extension that allows it a dual function as both a soluble chemo-attractant protein and an adhesion molecule. These dual roles allow immune cells that express the 7-TM G protein receptor CX3CR1 to track and bind to epithelial or endothelial cells that contain the membrane-bound form of the ligand (45). The separate chemokine domain, which is situated on top of the
extended mucin-like stalk, contains 2 cysteine amino acids that are separated by 3 other amino acids (CXXXC). This sequence represents an orientation that is therefore designated as the CX3C ligand-1 or CX3CL1.

The ligand’s receptor, CX3CR1, was previously identified as a G protein–coupled receptor (GPCR) that shares high sequence homology with monocyte attractant protein-1 and macrophage inflammatory protein-1A human chemokine receptors, which adds weight to the theory that the CX3CR1+ LPDC is myeloid derived. This distinct fractalkine receptor has been identified on only certain leukocyte populations, which include natural killer cells, some macrophages, lymphocytes and finally the intestinal dendritic cell (46) (41). The binding of the 95 kDa glycoprotein ligand, CX3CL1, to the CX3CR1 receptor present on the LPDC appears to assist not only in the adhesion of the LPDC to the epithelium but also to stimulate alterations in the LPDC cytoskeleton. These cytoskeletal modifications allow TED formation to occur through the organization of F-actin support structures which further permit the tight junctions to be penetrated by rigid dendrites, as previously discussed.

With regards to functional assays of the CX3CL1 ligand itself, the role of both membrane-bound and soluble forms is still being studied and defined within a spectrum of tissues and cell types. Concerning the circulatory system, researchers have found that endothelial cells, upon stimulation with soluble fractalkine ligand (s-FKN), respond with a direct dose-dependent increase of the intercellular adhesion molecule -1 or (ICAM)-1. These findings suggest a chemotactic communication signal where a cell becomes stickier or adherent as the ligand gradient increases (41) (47).
Upon further in-vivo investigation, where murine hearts were perfused with s-FKN protein, researchers found that the vascular endothelium also showed an increase in the mRNA and protein expression of ICAM-1. In order to truly determine the correlating receptor-ligand pathway, cultured endothelial cells were transfected with CX3CR1 siRNA so as to knockdown the transcription of the FKN receptor. These cells, following transfection, then ligand challenged with s-FKN and expression analysis was performed. The result of the siRNA knock down, in combination with the s-FKN challenge, showed that ICAM-1 expression was determined to subsequently decrease in direct correlation with the level of CX3CR1 siRNA.

Understanding the CX3CR1/CX3CL1 receptor-ligand pathway, in its ability to regulate expression of ICAM-1, provides researchers with an explanation as to why, when endothelial cells are stimulated with s-FKN, there appears to be greater attraction or adhesion of human neutrophils to the endothelial surface in vivo. These findings can suggest a parallel mechanism in the intestine, with the LPDC.

In addition to a direct correlative effect on ICAM-1 expression, current research has also found that endothelial cells display increased poly-phosphorylation of Jak2 and Stat5 upon CX3CL1 challenge. Likewise, it has been shown that when knocking down STAT5 with siRNA, significantly reduced ICAM-1 expression. From this endothelial model, much can be learned about signal processing and pathway activation. Knowing that inflammatory event results in increased production of CX3CL1 protein by the epithelium and eventually it’s ensuing cleavage in order to acts as soluble chemo-attractant chemokine (s-FKN), researchers are left to define these causative events and categorize elements of their activation in order to define the true cause and effect
mechanism of both the signaling cascade and subsequent binding dynamics of this system.

Further developing the idea that this soluble chemokine element or ligand, s-FKN, acts as a signal molecule capable of increasing the expression of adhesion molecules via the Jak/STAT pathway, one can begin to analyze an entirely new realm of study outside of the conventional trans-epithelial dendrite extension phenomenon that is commonly seen in the CXCL1/CX3CR1 axis. With the s-FKN cleaved form acting as both a chemokine and initiator of the Jak/STAT pathway, researchers can attempt to study the regulatory mechanisms and cellular interactions that occur within this novel receptor-pathway effector apparatus in different organ systems (47) (48).

Furthermore, the idea that the LPDC requires CX3CR1 to penetrate the epithelium suggests that it is possible that mutations within this receptor play a role in CD. With only half of CD patients possessing a NOD2 SNP, one must question whether mutations in CX3CR1 could effectively inactivate the LPDC and again “blind” it from sensing bacteria on the mucosa. A suggestion toward isolating the LPDC and genotyping the CX3CR1 allele among CD patients may begin to show some association and give more weight toward the LPDC as keystone element of the circuit.
IMAGING OF THE CX3CR1 LAMINA PROPRIA DENDRITIC CELLS

With such interest in the association between their focal location and function, a true in-vivo evaluation of the CX3CR1⁺ LPDC is necessary to determine their behavior in certain areas of the body, as these cells move toward regions of interest. Currently, dynamic imaging using fluorescence is leading the field because it has the ability to both monitor cells directly within their host tissue and evaluate the dendritic cell’s interaction with antigens and/or pathogenic organisms (25). Fluorescence possesses greater visual amplification of a target for labeling, such as the small trans-epithelial dendrite extensions, but also allows living cells to be analyzed. Beyond, the typical fluorescent-conjugated antibody, directed toward one or more antigenic epitopes on the CX3CR1⁺ LPDC, commonly:

1. CX3CR1
2. CD11b
3. CD11c

there has also been enormous in methods to express fluorescent reporters within cell-specific promoters of a gene. For example, the gene encoding the protein CD11c has often been used in a system where its upstream promoter will drive the expression of the chromophore-enhanced yellow fluorescent protein (EYFP). The capacity to visualize CD11c promoter expression, makes CD11c-EYFP a heavily utilized reporter system within the literature for not only many dendritic cells studies, but many myeloid-derived immune cell assays (49) (50).

However, not all reporter-expressing cells are surface CD11c positive. This concept, brought forth further reporter studies, in particular, more oriented toward the
CX3C-chemokine receptor 1 (CX3CR1). Currently, studies have evaluated both blood monocytes and myeloid-derived dendritic cells, of which the CX3CR1+ LPDC remains a progeny member. Conveniently, researchers have been able to develop a fluorescent reporter system to target these elusive cells by means of a CX3CR1 promoter driving the expression of the enhanced green fluorescent protein (EGFP) which has been referred to in literature as CX3CR1–EGFP (51). This distinct promoter/reporter combination has been particularly useful in in-vivo microbe challenge assays in that it allows one to monitor real-time events and to truly assess the pleomorphic nature and ever changing phenotype of the CX3CR1+ dendritic cell, as it alters its features and expression profile in response to stimuli and relative intestinal locations. (52) (53).

Theoretically, if all three combinatorial genetic identifiers (CX3CR1, CD11b and CD11c) could be assembled in a promoter-reporter-driven LPDC, researchers could not only study the location, expression pattern and cellular interactions that occur with these unmatched sentinels, but also use these three intracellular fluorescent makers as a way to efficiently isolate the cells in fluorescence activated cells sorting (FACS). FACS, although the currently accepted method of isolation, requires a relatively long drawn out tissue disaggregation process and the use of primary and/or secondary conjugated antibodies to detect extracellular protein epitopes of the CD markers. This method not only requires a tremendous time commitment, but also extreme delicacy and exactness in the antibody binding kinetics.

Even, after 24 hours of careful isolation and proper antibody binding, nearly half of the cells will have died and/or are in a state which does not resemble a healthy correlate to the cell it once was within the bowel. With such cellular distortion occurring
in the method of isolation, researchers in the field must begin to focus on a simpler, more efficient and less destructive way of extracting the CX3CR1\(^+\) LPDC from tissues, so that a more accurate understanding can be developed about these cells.

**Bacterial Interactions with the CX3CR1 LPDC**

The CX3CR1\(^+\) LPDC certainly utilizes an abundant number of pathways and other cellular entities within the lamina propria (54). It is clear however, this cell plays a pivotal role in sensing microbes that exist in close proximity to the intestinal epithelium (25). In fact, the mere presence of microbes not only increases the quantity of the CX3CR1\(^+\) LPDC present within the lamina propria of the terminal ileum, but also directly alters the phenotype of the cell, in that it induces the extension of TEDs toward the nearby organisms (Figure 7) (36). Beyond the simple change in number of the CX3CR1\(^+\) LPDCs within the lamina propria and in their ability to extend TEDs, the inflammatory expression profiles of the LPDC are also altered when in contact with microorganisms. Stimulation of CX3CR1\(^+\) LPDC with fractalkine ligand, after previous exposure to LPS, the DC appeared to increase the release of IL-6 and TNF-\(\alpha\) leading preferentially to Th1/Th17 CD4 T cell differentiation (55).
Figure 7. Alteration in the DC TED extension following microbial stimulation. Fluorescent images depict the extension of dendrites following bacterial challenge, while the bar graph quantifies the dendrites per villus area. SPF represents a wt CX3CR1 DC, while GF represents a mutant DC. The partial figure was taken from reference (36).
MIGRATION PATTERN OF THE LPDC

The myeloid-derived macrophage and DC express a large repertoire of pattern recognition receptors (PRR) (35). Among the PRRs are the Toll-like receptors (TLRs), which are extracellular trans-membrane proteins capable of augmenting inflammatory gene expression upon ligand binding. TLRs are usually distinguished from the intracytoplasmic muramyl dipeptide receptor, nuclear oligomerization domain 2 (NOD2), in that NOD2 is found within the cell and capable of carrying out multiple types of effector functions. These immune functions involve everything from phagocytosis, to intracellular bacterial transport, to gene regulation through the NF-κB pathway, and finally the more mechanical system of dealing with microorganism disposal by means of the autophagy pathway (56) (57).

The NOD2-containing CX3CR1+ LPDC population modulates the immune responses directly in the mucosa and serves as a first line barrier against invading enteropathogens (4). The precise subepithelial location of the CX3CR1+ LPDC and the arsenal of microbial sensors is what validates this cell species as a professional APC, in that its primary role is to sample the organisms within the lumen via TED extension, assimilate the information and direct a focal immune response within the crypt-villus axis (20).

This unique CX3CR1 receptor allows for extremely close apposition of the LPDC to the intestinal epithelium, so as to properly position the APC as close to the barrier as is physically possible. In addition, the only known ligand for this dual function chemokine/adhesion receptor is CX3CL1, which the epithelium expresses on the basal laminal surface and can adjust depending on the local inflammatory state of the mucosa (26).
As seen in Figure 8, the CX3CR1\(^+\) LPDC has maneuvered itself significantly closer to the sub-epithelial borders than the CX3CR1\(^-\) CD103\(^+\) dendritic cell, which remains in the core of the villus behind the CX3CR1\(^+\) LPDC (35). This figure depicts the location of the two types of dendritic cells located in the lamina propria (the CX3CR1\(^+\) cell and the CD 103\(^+\) cell) and depicts different functions based on these distinctive positions.

**Figure 8. Locations of the two types of intestinal dendritic cells** and the distance they are found away from the epithelium. In addition, the graphs of the figure describes the number of cells found within each villus. The partial figure was taken

Although the CD103\(^+\) DC does participate in antigen presentation, its main responsibility is to migrate back to the mesenteric lymph node (MLN) and present that antigen to the immune-cellular entities (B and T-cells) that populate the MLN. This migration by the CD103\(^+\) DC, in a sense, acts to prime MLN cells and display to them what the DCs are seeing at the luminal border. This reverse functional migratory pattern toward the lymph node is not seen in the CX3CR1\(^+\) CD103- LPDC, in that it maintains
its position in order to continuously sample luminal contents by way of TEDs and communicate to the migratory cells (CD103+ DC and tissue macrophage) assistance if needed (26) (58). In summary, the CX3CR1+ LPDC obtains microbes through the TED, digests them in the phagolysosome, binds MDP to NOD2, activates the NF-κB and presents microbial remnants via MHC-II to the CD103+ DC for transport to the MLN.

The close positioning of both groups of intestinal DCs, along with their specifically defined roles, suggests future studies for researchers relating to the isolation of both groups of cells in order to combine them ex-vivo under particular stimulatory conditions so as to manipulate the possible communication systems that exist between them and better understand their positions in gut immuno-modulation at the epithelium. Providing the CX3CR1+ LPDC with MDP may elicit a secretory product that directly communicates with the CD103+ DC. Knocking out NOD2 or CX3CR1 in these cells and then providing that same stimulus may show alterations in the CX3CR1+ to CD103+ communication, again providing insight to Crohn’s Disease and the coupling effect of mucosal dendritic cells of the intestine.

**Migration of the LPDC Toward the CX3CL1 Protein**

To this point, this review has discussed the immunologic role and some of the stimuli which act on the LPDC of the gut; with that, the exact migratory mechanisms of the CX3CR1+ LPDC can be discussed. The expression of chemokines by the epithelium of the intestine has been suggested to assists in developing the proper chemo-attractive chemical gradient necessary to direct white blood cell migration to the appropriate target.
zone within the mucosa of the intestinal wall (59) (60) (61) (62). Until recently, many models have held firm with the conventional methodology that during leukocyte migration there is a certain or distinct order of events necessary for a white blood cell to remove itself from circulation and eventually enter at a specific tissue focality within the body. It is wondrous how single cells, moving quickly through the circulatory system can stop at a small region of endothelium and extravasate into surrounding tissues.

The textbook diagram of this blood to tissue migration mechanism essentially depicts a region of dilated vascular tissue where the release of chemokines and activation of certain receptors creates a “sticky zone.” This sticky zone is created in order to slow the leukocytes that are rolling along the endothelial luminal wall within the circulation to a flow rate that allows these cells firmly to bind via ICAMs, integrins and selectin proteins (Figure 9) (63).

**Figure 9. Schematic portrayal of factors involved in leukocyte recruitment in LPS-induced TLR-mediated inflammation.** Upon activation of endothelial cells and leukocytes pro-inflammatory factors are released and the exposure of cell-surface adhesion molecules (selectin, ICAM-1/VCAM-1, integrins) leads to leukocyte rolling and eventually to leukocyte adhesion. PECAM-1 and CD99 are involved in the subsequent leukocyte transmigration. Figure taken in its entirety from: *Cardiovasc Res.* 2003 Oct 15;60(1):49-57. *Molecular basis of endothelial dysfunction in sepsis. Peters K, Unger RE, Brunner J, Kirkpatrick CJ.*
In the end, “this exquisite specificity of leukocyte homing, determined by a combinatorial “decision processes” involving a multi-step sequential engagement of adhesion and signaling receptors,” was thought to be how most, if not all, of the general pathways within chemokine-directed migration patterns worked for leukocytes (64).

However, this conventional pathway of complex, multi-protein and multi-signal induction systems involved in slowing the rolling leukocyte is not the case for cells that recognize fractalkine. CX3CL1 is what seems to be the first chemokine motif (CX3C), that appears to have the direct and exceptional capacity to combine leukocyte attraction, capture, firm adhesion, and activation of the cells without the assistance of integrins (58) (26). These dynamic studies which have evaluated the ability of CX3CL1 to adhere to cells expressing CX3CR1, have also shown that the G-protein-coupled component, which lies intrinsically within the receptor, can be activated in order to permit rapid adhesion, much faster than VCAM/integrin systems (59). This “rapidity” is thought to not only play a role in the delivery of cells but also the delivery of the TED across the epithelium when basal levels of micro-organisms exceed physiologic levels.

**Cytoskeletal changes in Migration and those that occur with NOD2 and CXCR1**

The mechanism involved in dendritic cell migration as it pertains to rearrangement of the cytoskeleton is still not well understood. However, studies have indicated that the Wiskott-Aldrich Syndrome protein (WASp) and the actin organizing actin-related protein 2/3 complex (ARP2/3) are necessary for the initiation of filaments and the development of podosomes. These filaments and podosomes act to extend
adhesive structures located at the leading edge of migrating cells in order to further pull the cell in one direction or another. Dendritic cells that lack the WASp complex do not assemble leading edge podosomes. Without these podosomes, DC migration comes to a halt.

Additionally, complex micro-injection studies have revealed that proper podosome assembly, and thus polarization of the leading edge also require assistance of the universal RHO-family GTPases: Cdc42, Rac, and Rho, thereby providing a link between the sequential protrusion, adhesion and pulling activity required for DC cellular movement (60). Beyond the basic WASp-ARP2/3 collective mechanism of dendritic cell migration, the NOD2 receptor activation pathway has also been evaluated regarding its role in alterations of the cytoskeleton (61).

As for CX3CR1, little is known about the mechanism of extension of the trans-epithelial dendrite within the LPDC; however, recent research has helped in determining the type of interaction that occurs between the TED and epithelial cell upon penetration. Understanding that, in order for the LPDC to function properly in an intestinal system where the lumen contains highly antigenic and often toxic materials, the dendritic cell must be able to extend its trans-epithelial dendrite across basal lamina and between enterocytes in a way that protects the internal homeostasis of the organism. To do this, regions of the dendritic cell appear to express tight junction proteins in order to penetrate gut epithelial monolayers and subsequently sample bacteria that have occupied regions close to the luminal side of the mucosal wall. It is because these unique LPDC’s express tight-junction proteins such as occludin, claudin 1 and zonula occludens 1, that the
seamless cellular integrity of the epithelial barrier is preserved and maintained in a properly functioning physiologic and immunologic manner (62).

**Figure 10. DCs cross the filter and infiltrate the epithelial monolayer. (a–c)** D1 cells were added to the compartment facing the basolateral side of the Caco-2 monolayers. After 5 h of co-culture, monolayers were incubated with bacteria added on the apical side; the TER was unchanged throughout the whole experiment. Filters were then fixed and processed for transmission electron microscopy. (a) D1 cells crossed the filters and infiltrated between epithelial cells (EC) opening their TJ openings without changing microvilli organization (magnification: x15,000). (b) and (c) The boxed region in a was magnified further to reveal TJ-like structures, indicated by arrowheads, between DCs and Caco-2 in two serial sections (magnification: x47,000). Figure taken in its entirety from Rescigno M, Nature Immuno 2001 Apr;2(4):361-7
Figure 11. Scheme of the events occurring during a bacterial infection.
Under resting conditions, infiltrating DCs establish loose contacts with preexisting epithelial TJs. Upon bacterial infection, DCs are recruited from the blood and activated, probably via epithelial cell signals. They up-regulate the expression of occludin, which in turn allows DCs to compete for epithelial occludin and open up the TJs, like a zip. Infiltrating DCs then face the gut lumen and can directly sample bacteria. Bacterial components such as LPS trigger the reorganization of TJ proteins via up-regulation of ZO-1 and the disappearance of occludin, thus allowing the DCs to detach from junctions with epithelial cells and to migrate into the draining lymph nodes. Figure taken in its entirety from Rescigno M, Nature Immuno 2001 Apr;2(4):361-7

Figure 10 depicts an electron micrograph, where one can clearly see an intact membrane system between the LPDC and epithelium. Figure 11 depicts the sequence of expression of particular tight-junction proteins (occludin, claudin 1 and zonula occludens
in order for the LPDC to extend TEDs in a zipper-like manner, while maintaining an impermeable mucosal barrier.

These basics of this dual cellular interdependent mechanism, which maintains the impermeable mucosal barrier, begins with the DC being recruited to the focus of inflammation, most likely by inflammatory chemokines such as the epithelial soluble fractalkine form of the CX3CL1 ligand and/or macrophage inflammatory protein 3α (MIP-3α) and chemokine (C-C motif) receptor 6 (CCR6). Commonly these types of proteins are secreted by epithelial cells when placed under “stress,” whether pathogenic or hypoxic in nature (63) (64). Next, DCs are induced to up-regulate tight junction proteins and institute tight junction multi-meric structures alongside epithelial cells. These multi-cellular interactions occur in what appears to represent a “zipper-like” process, where tight junctions are released in front of the TED and then re-created behind the leading edge of the extension in order to take up antigen and still maintain the impermeable barrier at any given moment. The exact mechanism that allows the lamina propria dendritic cell to destabilize enterocyte binding is still being studied; however, Figure 11 describes the proposed sequential steps to the best of our knowledge.

It is clear from the current research that a better model system is needed in order to further understand this dynamic “zipper-like” movement between the LPDC and enterocyte. With the development of self-organizing epithelial cell lines and sub-mucosal supporting structures, researchers will soon be able to add DC cell populations to these “semi-ex-vivo” systems in order to study TED extension dynamics in a more controlled step-by-step method. As is clear with most systems being studied today, a well
understood model possesses enormous insight into what is most likely occurring within the organism.

THE EXTENDED FUNCTION OF NOD2 IN LPDC

Beyond its role in chronic inflammation within a transplanted organ, the CX3CR1+ LPDC also appears to play a significant role in native tissues, particularly within the intestine. Here, within the LPDC, the nuclear oligomerization binding domain (NOD2), which acts as an intra-cytoplasmic receptor for the bacterial cell wall component, muramyl di-peptide. It has been shown, that mutation of the NOD2 gene is associated with an increased risk of developing Crohn’s Disease (relative risk 44) (65). This is thought to result from an inability of the antigen-presenting cells to detect bacteria properly, due to the lack of a functioning NOD2 receptor, but also because the receptor may play a communicative role to downstream pathways in both inflammation pathways such NF-κB and bacterial handling systems such as autophagy (66) (67) (68) (69). Initially, NOD2 was thought to function within a phagosome by means of sensing the MDP ligand at the LRR region and then subsequently altering the receptor formation in order to induce downstream activation binding events. Following this, the receptor-harboring phagosome was found to migrate toward the plasma membrane in order to interact with and successively activate the NF-κB pathway (Figure 12).

Here, at the membrane, the functional NOD2 protein associates with the caspase activation and recruitment domain of the RIP-like interacting caspase-like apoptosis regulatory protein kinase (RICK). The RICK/RIP2 complex at this time then activates nuclear factor NF–κB in epithelial cells and macrophages (39). In contrast, the NOD2
mutant 3020insC, as seen in Figure 12, contains a frame-shift single nucleotide polymorphism, which shows impaired ability to activate NF-κB. (70)

Figure 12. Localization of NOD2 in wild type and mutant settings. (B, 1) GFP-NOD2 wild type and the three main NOD2 mutations that are associated with CD (GFP-NOD2 3020insC, GFP-NOD2 R702W, and GFP-NOD2 G908R) were transfected in COS7 cells. Only NOD2 3020insC failed to co-localize with the plasma membrane, whereas the two other NOD2 mutant forms still showed membrane association (arrows). Bar, 20 μm. (2) Expression of NOD2 mutants and NF-κB activation were determined and compared with untransfected and nonstimulated HEK293 with MDP-LD, as described in 220. Figure taken in its entirety from Barnich J Cell Biol. 2005 Jul 4;170(1):21-6
NOD2 protein expression has been discovered in numerous cells ranging from intestinal epithelial cells (enterocytes and Paneth cells) to myelo-derived leukocytes (71) (9) (72). The NOD2 receptor, as previously stated, recognizes and reacts to the bacterial component muramyl di-peptide L-Ala, D-Glx (MDP-LD) via its COOH-terminal leucine-rich repeat domain (73). However, when the NOD2 receptor carries the Crohn’s disease associated 3020insC mutation, the receptor is no longer capable of recognizing the MDP ligand and initiating the NF-κB response pathway by way of migration to the membrane (74).

Beyond the NOD2 protein-protein interaction with the NF-κB complex, there has been recent understanding, using promoter sequence analysis (Figure 13), of an additional intricate positive feedback system where NF-κB actually enhances the transcription of the NOD2 gene, via a binding site within the NOD2 promoter itself (75). This suggestion within the literature that NF-κB can somehow provide a transcriptional feedback loop on NOD2 has been somewhat substantiated in that the NOD2 single nucleotide polymorphism appears to alter the dendritic cell’s NF-κB expression profile in some studies (21). In these studies, researchers used monocyte-derived dendritic cells from both NOD2 wild type and NOD2 mutant patients and challenged them with MDP and LPS in order to monitor effector functions based on cytokine expression. Their findings correlate with what is seen clinically in that there was an improper over expression profile of Th1 priming cytokines and an abnormal ratio of IL-12 to IL-10. Over activation of the Th1 pathway leads to tissue targeted destruction, which again, is commonly seen in Crohn’s Disease. The cytokine profile is alternatively dichotomous to the normal response of the bowel to bacteria, where the human tissue of the gut is spared.
Additionally, when stimulated or challenged with *Salmonella*, the NOD2-defective DC shows considerably reduced expression of the cytokines IL-8, IL-12 and IL-10 compared to the NOD2 wild type leukocyte. In addition to these depressed interleukin profiles, the inflammatory cytokines TNFα and IFNγ were found to be...
improperly over expressed by the NOD2 mutant DC under direct *Salmonella* stimulation. Interestingly, all of these findings within the DC correlate with what is seen in the intestinal tissue in Crohn’s patient biopsies during both basal and hyper-inflamed flare-up states (76). These results appear to support a NOD2 defect in the bone marrow derived immune cells with over expression of TNFα and the reduction of regulatory interleukins (IL-8, IL-12 and IL-10).

**Bacterial Uptake by CX3CR1 LPDC**

Understanding that the CX3CR1$^+$ LPDC has the capacity to access the lumen by way of trans-epithelial dendrite extensions was a major finding in gut immunology (75) (77). However, the mechanism by which these extensions physically interact with microorganism is not entirely understood. Research has shown the dependence of the TED extension on the CX3CL1 ligand and that the process of uptake involves the “normal phagosome vesicle” formation in order for bacterial acquisition to occur.

![Figure 14. Depiction of DC TED extension in acquisition of microorganisms as compared to a leukocyte without TED extension during episodes of colitis. (79)](image.png)

This would suggest that the only true mechanical difference between an LPDC and an intestinal tissue macrophage is the ability to form TEDs and the ability of the DC to sample the contents of the lumen of the bowel (Figure 14).
Recent experiments in the field of gut APC immunology, involving the organism *S. typhimurium*, have led to further understanding about this uptake mechanism. What makes *S. typhimurium* such a unique organism and perfect candidate for this study is that it possesses a virulence factor (type 3 secretion systems) which allows it to escape from the phagocytic vesicle prior to the fusion of the phagosome with the acidic/oxidative lysosome. This allows *S. Typhimurium* to safely reside within the intracellular compartment of the immune cell and has served as a model to understand a more about the capacity of the CX3CR1⁺ LPDC to deal with microbes that can persist intracellularly (78).

These *S. typhimurium* challenge studies with the LPDC found that early uptake and infection of the host by *S. typhimurium* occurred mainly in LPDCs that possess the CX3CR1 receptor. The ability of the LPDC to deal with the intracellular microbial threat appeared to be dependent on the capacity of the DC to develop intact functional intracytoplasmic vesicles. This LPDC vesicle formation relied on additional downstream pathways in addition to the classical TLR4-dependent myeloid differentiation primary response gene-88 (MyD88) vesicle system, which macrophages typically use to deal with both extracellular and intracellular organisms (79). Thus, the LPDC not only possess the unique quality to extend TEDs in dealing with microbial challenge, but can also remove the organism through a different disposal system than other antigen-presenting cells. Previous research has argued it is simply the delivery of the TED to the apical surface of the epithelium in combination with the broad spectrum of pattern recognition receptors that makes this LPDC unique, not that it deals with bacteria in some new undiscovered way. Recent focus on the *S. typhimurium* challenge group answers these concerns, with
yet another important result in the MyD88 system of the DC, again showing this cell is different from the intestinal macrophage.

Seeing that the LPDC is normally capable of dealing with invasive bacteria, researchers began to study the role of autophagy in dealing with intracellular organisms and found that the activation of NOD2 by muramyl dipeptide induced autophagy in DCs via the NOD2 receptor interacting with the well-known intermediate serine-threonine kinase-2 (RIPK-2) and additional autophagy-related proteins such as ATG5, ATG7 and ATG16L1 (27). It has been argued by some that this mechanistic protein-protein interaction between NOD2 and ATG16L1 occurs independent of both the RIP2 adaptor protein and the classic NOD2 downstream activation of the transcription factor NF-κB. As an alternative, it has suggested that this NOD2 activation of ATG is instead occurring through more of a sequestration mechanism where NOD2 recruits the nucleating protein (ATG16L1) from the cytoplasm to the plasma membrane leading to the autophagy vesicle formation. Once located at the plasma membrane, eventually wrapping itself around invading bacteria, the complex of NOD2/ATG16L1 and plasma membrane then begin to form the double membrane autophagosome that defines autophagy (80) (81).

Those favoring this NOD2/ATG16L1 interaction explain that truncated forms of the NOD2 receptor, as seen within the frame shift mutation 3020fs, and/or truncation with ATG16L1, lead to inactivation of this interdependent pathway and an inability to deal with phagocytosed pathogens. These findings strengthen the argument for a genetic link between CD development and a relationship between two functionally-associated genes, NOD2 and ATG16L1. The spectrum of pathology and severity within CD could
be explained by the fact that mutations might occur in anyone of the multiple genes and/or combination of genes associated with the disease.

**Autophagy’s Role in the Paneth Cell of the Intestine**

Autophagy in Crohn’s Disease was initially thought to be more important within the epithelium of the intestinal mucosa rather than the bone marrow-derived immune cells that populated the lamina propria. Epithelial studies in transgenic mice have indicated that ATG16L1 plays multiple roles in inflammatory bowel disease as it relates to the mucosal wall. These ATG16L1 functions are seen within the Paneth Cell (PC) deep within the crypt epithelium (25). Here, the autophagy-nucleating protein, ATG16L1, when mutated, appeared to be linked to (1) an abnormal appearing and (2) reduced functioning Paneth cell within the terminal ileum (37). This abnormality was apparent on a gross microscopic level in that the granules that are typically found within the Paneth cell and function to exocytose their contents (antimicrobial products—α-defensins, lysozyme, and secretory phospholipase A2) were significantly deceased in quantity. This would suggest that a decreased quantity of anti-microbial agents would lead to increased accumulation of microorganisms, something that is seen in CD.

In addition, the granules that were present appeared morphologically atypical, while the cytoplasm of the Paneth cell appeared to contain large regions of diffuse cytoplasmic lysozyme staining, suggesting the release of once sequestered contents. From this descriptive evaluation of the PC phenotype, as it relates to a mutation in the ATG16L1 gene, researchers suggested that autophagy influences the ability of the Paneth cell to function properly in its role of reducing bacterial bio-mass within the gut.
Now knowing that the autophagy pathway intersects the lysosomal pathway and, additionally, that granules or compartments involved in the secretory system are essentially a specialized form of lysosome, it is reasonable to suggest that autophagy might be directly involved in the “biosynthesis or “quality control” of granules within the Paneth cell. On the other hand, the fewer granules found in these paneth cells might be a result of exocytotic exhaustion due either to (1) an inability to deal with increased basal bacterial levels, requiring the constant release of anti-microbial peptides or (2) the loss of epithelium, due to a constant inflammatory state, leading to a more immature and less productive Paneth cell (4) (82).

**Paneth Cell Maturation and Wnt**

The Paneth cell of terminal ileum, as discussed earlier, is located at the lowest point within the crypt. There, it plays a pivotal role in regulating bacterial load within the lumen and controlling those organisms that appose the epithelial wall of the intestine. These distinct anti-microbial or natural antibiotic-producing cells, like other cells of the small intestinal crypt-villus axis, eventually mature from a compartment of progenitor cells that also reside within the crypt, near the +4 region located Lgr5+ intestinal stem cell (83). The progenitor cells, which will eventually develop into Paneth cells, are under to the control of the canonical Wnt signaling pathway (73).

This canonical Wnt system communicates via a Wnt ligand binding to a Frizzled and LRP receptor leading to intracellular transduction via the β-catenin → TCF4 pathway. Activation of the Wnt pathway results in the formation of an intracellular β-
catenin-TCF4 complex, which translocates into the nucleus, where TCF4 acts as a transcription factor to control the expression of several downstream target genes. This molecular ligand interaction helps maintain the undifferentiated state of the progenitor cells in that it prevents the progression of the progenitor toward the enterocyte pathway.

**Figure 15. Location of the Paneth cell.** Staining with phloxine tartrazine to detect human defensin 5 in terminal ileum. (Left) Represents a Crohn’s Disease patient with depressed levels of TCF4, which correlate to reduced Human Defensin 5 labeling, the primary alpha defensin antimicrobial peptide of the terminal ileum. (Right) Represents a non-CD patient with normal TCF4 levels and subsequent normal HD5 levels. Researchers in the CD field have argued for years that this simple observation of reduced HD5 in CD patients is what signifies the initiating event of bacterial accumulation and gut inflammation. The partial figure was taken from Wehkamp Gut. 2004 Nov;53(11):1658-64.NOD2

This Wnt pathway however plays an additional role, in that it promotes differentiation and maturation of the Paneth cell (84) (85) (86). This dichotomy between
progenitor maintenance and Paneth cell derivation is critically dependent on transcription factor 7-like 2 (TCF4), which activates many of the genes necessary for Paneth cell function such as matrix metalloprotease MMP-7 and Paneth cell α-defensins (Human α defense 5 and 6) (86). Without proper activation of TCF4, expression of αHD5 remains impaired (87) (88). Recent studies, evaluating TCF4 control of αHD5 in CD patients has shown a correlation between the reduction in TCF4 and a depletion of αHD5, suggesting Wnt pathway abnormalities could indeed explain lower levels of the anti-microbial peptide within the Paneth cell (Figure 15).

**HUMAN DEFENSIN 5**

Until now this review has discussed the CX3CR1+ LPDC, the epithelium, the Paneth cell and the communication that takes place between the two compartments (lamina propria and epithelial compartment). In order to begin to come full circle, one must discuss the anti-microbial peptide effector which fulfills the role of chemical protection at the gut barrier. Understanding as to how alpha defensins, particularly αHD5, play an important role as “natural antibiotics” at the mucosal/epithelial barrier, it comes as no surprise that their diminished expression may permit microbial invasion of the intestinal wall. This invasion may secondarily lead to the induction of inflammatory events that may be key initiating processes which further induce the “Crohn's Disease flare up” that is seen clinically. With diminished αHD5 levels correlating with NOD2 and ATG16L1 mutations in patients, one might ask what is it about αHD5 itself that provides such an important function (89) (90). To put it another way:
Why is it, that a defect in αHD5 leads to bacterial invasion, when there are other anti-microbial agents such as phospholipase-A2, lysozyme and inorganic peroxides present within the Paneth cell, which by themselves are capable of killing bacteria?

Human alpha Defensin 5 (Entrez Gene ID: 1670) or αHD5, DEFENSIN, ALPHA, 5; DEFA5, DEFENSIN 5; DEF5 (8p23.1), was cloned in 1998 using a 3-prime RACE protocol (91). In this study, αHD5 was found to be more highly expressed in inflamed vaginal epithelium. However, αHD5 was first identified in 1992 using genomic clone analyses (89). This particular study found that αHD5 is highly expressed in Paneth cells of the small intestine. This localization study defined it as the first example of an anti-microbial peptide gene expressed in human epithelial cells. Further study of the location of the peptide was performed using microscopy, which led researchers in 1997 to determine that the protein αHD5 was highly concentrated within Paneth cell granules of the small intestine, particularly the terminal ileum (92). It was thought that since Paneth cells release their granules adjacent to the mitotic cells (transit amplifying region) of the intestinal crypts, αHD5 could protect this highly important and fragile cell population against invading organisms and possible parasitization by microbes (91). After all, without a protected Lgr5+ stem cell, the gut epithelium is left without cellular reproductive activity.

Later researchers discovered that αHD5 existed in a precursor form within the Paneth cell granule and that release of this granule appeared to be related to stimulus by the neurotransmitter acetylcholine or certain bacterial cell wall components, such as lipopolysaccharide or peptidoglycan. Upon secretion into the crypt, the zymogenic pro-
form was activated following cleavage by trypsin (90). This zymogenic form is important in that it stabilizes the pre-peptide and allows it to be stored for later use even while within the reducing environment of the cell. Processing of the pro-form to the mature structure comes at a cost, in that recently it has been found that among NOD2 mutant-individuals, the peptide is not cleaved by trypsin and remains stuck in a complex with the protease trypsin/trypsinogen, preventing it from effectively acting as an anti-microbial agent (91).

The defensin family acts through a mechanism where the peptides form dimeric pores within the bacterial cell wall by coupling with another defensin peptide (93). Within the peptide, 6 cysteine residues cross-link to assemble three disulfide bonds that are crucial for the function of the peptide in forming a pore. In addition to the three disulfide bonds, there is one Gly residue, which is required for the peptide to form the distinct beta-bulge within the chain that defines the defensins. Finally, a salt bridge formed by a pair of oppositely charged residues (Arg/Glu) act to further stabilize the defensin within the caustic intestinal lumen so that it will be properly processed from its zymogenic to mature form. These specific residues are conserved throughout all alpha-defensins and permit the peptides to function as an epithelial based anti-microbial agent within the gut (94).

The unique behavior of this pore-forming antimicrobial peptide has sparked interest in the development of this agent as an antibacterial therapeutic; however, the utilization of the zymogenic pre-form within a delivery capsule and/or via intravenous delivery has not been well established. Presenting this peptide in an inactive form or in a way that is sub-optimal prevents high levels of activity against bacteria. This
ineffectiveness, in addition to the high cost of generating peptide-derived agents (i.e. insulin) prevents αHD5 from coming to market at this time. More molecular and discrete segment analysis of the peptides need to occur in association with bacterial kill assays in order for there to be any value in developing this agent as a potential antimicrobial peptide drug in clinical medicine. In fact, after understanding the structure-function relationship and being able to generate a suitable stable form, application of the antimicrobial peptide within other organ systems (skin, endovascular and CSF) could prove to be beneficial in infections (necrotizing fasciitis, sinusitis, otitis media, sepsis, meningitis etc).

**THE WNT5A LIGAND**

As Paneth cells operate and mature via the Wnt pathway activation and that αHD5 expression is highly regulated based on TCF4 (a Wnt directed transcription factor) activation within the Paneth cell, the final topic of this literature survey will focus on communications that occurs at the interface of the two compartments that exist within the gut wall (the lamina propria compartment and the epithelial compartment). To approach the topic of communication, a brief understanding of Wnt as an inflammatory ligand is needed, since Wnt is often thought of as a cell developmental ligand. The Wingless-type MMTV integration site family, member 5A or Wnt5a (NCBI Gene Entrez ID number 7474) (chromosome 3p21-p14) belongs to a group of genes which code for ~40 kDa glycoproteins that contain cysteine-rich regions (21 conserved cysteine residues), and typically function as secreted or ligand-like growth factors (NCBI). In 1990, the particular ligand, Wnt5a, was discovered by researchers who were selectively using
degenerate PCR to search for genes similar to Wnt1 in mice (95). The 4.1 kb gene was cloned and shown to be the ligand for the human Frizzled-5 receptor (FZD-5); further validating the claim that the peptide termed Wnt5a belonged to the Wnt family (86). However, it was not until 2008 that the effect of the Wnt5a ligand on cellular phenotype and expression (trascriptome and proteome) were thoroughly evaluated.

It was discovered that cell-autonomous mechanisms permit Wnt5a to “control cell orientation, polarity, and directional movement in response to positional cues from chemokine gradients” (96) Furthermore, these studies indicated that upon stimulation of a melanoma cell line with the Wnt5a ligand, a composite of the structures began to form what appeared to be an intracellular complex within the cell line (97). Within this complex actin, myosin IIB and the human frizzled 7- pass-trans-membrane receptor accumulated at the intracellular border leading to contraction of the membrane. At this location, below the membrane where the complex was nucleated, appeared to be the initiating point where alterations in cell polarity, and/or directional movement occurred under induced Wnt5a chemical gradients (98). These findings suggest that Wnt5a could lead to alterations among cytoskeletal elements within a cell and modify the nature of the plasma membrane of the cell, beyond simply that of maturation or development.

**WNT SECRETION BY MYELOID-DERIVED CELLS**

Beyond the conventional Wnt ligand theory, where Wnt gradients induce alterations in cellular maturation and/or polarity, recent investigations have led researchers to discover yet another important, albeit unique, utility of the Wnt5a protein. This novel function appears to involve an inflammatory communication circuit that exists among myeloid-
derived antigen-presenting cells and T-cells (99). Current research suggests that the activation of TLR2 by way of mycoplasma challenge and/or TLR4 stimulation, via its ligand LPS, leads to an increase in the expression (transcription) of the Wnt5a secretory protein through a multi-step TLR signaling pathway. This release or secretion of Wnt5a, and subsequent binding to its frizzled receptor, results in the activation of Iκβ-α to NF-κB within a target cell.

**Figure 16.** Dendritic cells secrete Wnt5a upon stimulation of pattern recognition receptors via the NF-κB pathway. Toll-like receptor–induced, NF-κB–mediated activation of human antigen-presenting cells (APCs) leads to transcription of WNT5A, which, released or cell surface bound, is able to interact with FZD receptors (eg, FZD5). WNT5A and FZD5 regulate the microbial-induced IL-12 response in an autocrine manner and thereby prime specifically activated T lymphocytes for IFNγ release. In addition, since lymphocytes also express FZD5, a paracrine effect of WNT5A on these cells directly regulating the IFNγ response is possible. Reagents used to develop this model are indicated by and in the affected pathways. Heine H, Blood. 2006 Aug 1;108(3):965-73. Epub 2006 Apr 6.

This NF-κB pathway activation materializes as it translocates to the nucleus, further augmenting Wnt5a gene expression. Challenge studies performed on the antigen-presenting cells indicate that the ligand Wnt5a and its receptor, Frizzled-5, alter IL-12
production in conjunction with TLR stimulation. The acute increase in IL-12 production further assists in priming the T-cell in order to produce IFNγ (Figure 16).

Taken overall the data suggests that significant amounts of Wnt5a are secreted by myeloid-derived cell entities when challenged with a bacterial signal, commonly seen in septic patients (100). The autocrine and paracrine method of secreted activation, although heavily studied, is still far from defining the Wnt pathway responsible for the cellular inflammatory effect. This ambiguity results from the fact that diverse signaling pathways may be triggered subsequent to Wnt5a binding and activating the Frizzled 5 transmembrane receptor.

One of these pathways, the canonical Wnt signaling pathway, is known to progress through a mechanism that results in the freeing of the β-catenin protein from its sequestering destruction complex (axin/GSK-3/APC) which promotes the proteolytic degradation of β-catenin by the ubiquitin/proteosomal pathway. The non-canonical pathway progresses by acutely increasing the level of intracellular Ca2+ di-cation. Both alternative pathways have been studied with regards to the Wnt5a ligand activation system. On balance, the data favors the non-canonical Wnt signaling pathway when taking the inflammatory circuit into consideration, in that the effects of increased Ca2+ have been seen downstream. Upon rapid increase in intracellular Ca2+ one of the downstream calcium-dependent kinases (CaMKII) appears to further stimulate the nuclear translocation of the transcription factor, nuclear factor of activated T-cells (NF-AT), a specific dendritic cell life cycle regulatory factor (101).

Wnt5a appears not only to act in altering cell polarity and inflammatory communication between bone marrow-derived immune cells but also to act as a ligand to
stimulate the Wnt-directed Paneth cell. This communication network between gut immune cells and the epithelium, although a nascent field, appears to provide possible answers to many complex questions.

A CONCLUSION OF THE LITERARY REVIEW

Since the mid 1990’s there has been an explosion in research surrounding the ability of the dendritic cell to penetrate the epithelium and sense bacterial components within the gut lumen. Since then, there have been numerous publications on the mechanisms of penetration and the communication that takes place between the bone marrow-derived cells of the immune system and the epithelial cells of the small bowel. However, outside of the current mouse models, which lack the ability to explain the DC-to-Paneth cell augmentation of αHD5 because mouse models do not have an αHD5 gene, researchers are left to observational studies in humans. Beyond the monocyte-derived dendritic cells that are commonly used in human studies, researchers are left trying to establish a model to examine method the communication between the CX3CR1^+ LPDC and epithelium.

A possible approach to circumvent pure observational studies would be intestinal mucosa organotypics. This organotypic system could be constructed using methods developed by the Clever laboratory in isolating Lgr5^+ intestinal stem cells and growing self-sustaining crypt-villi structures. Genetic alterations of these free structures could assist in determining the basal levels of the crypt as it pertains to stem cell turnover and anti-microbial peptide development. Once these isolated studies have been performed, researchers could then add a pure population of the CX3CR1^+ LPDC to determine its role.
in crypt function. Finally, adding microorganisms to the system under both control and NOD2 knock out systems could explain why these genes, in particular, play such a pivotal role in epithelial immunology and Crohn’s Disease. Once, this semi-synthetic, compartment system becomes a viable option, studies will become more relevant and less associative and/or speculative with regards to causes in barrier failure in CD.

THE CURRENT STATE OF CROHN’S DISEASE

At the present time the prevalence of Crohn’s Disease is 149 per 100,000 people, which easily places it among the most common serious, non-infectious, bowel pathology in humans. Beyond its debilitating progression through the development of ulcers, granulomas, fistulas and scarring within the bowel, the disease can be deadly in advanced cases. In the past, the disease was thought of as an “intestinal disorder” which could be dealt with by treating the bowel directly with steroids and resecting areas of necrosis or severe fibrosis. However, currently, the Crohn’s and Colitis Foundation for American recognize and publicize the role of autoimmune-directed inflammation of the bowel, in particular the terminal ileum, when discussing the development of CD. Moreover, the foundation does recognize mutations in NOD2/CARD15 as increasing susceptibility:

“Recently, an important breakthrough was achieved when the first gene for Crohn’s disease was identified by a team of IBD investigators. The researchers were able to pick out an abnormal mutation or alteration in a gene known as NOD2/CARD 15. This mutation, which limits the ability to recognize bacteria as harmful…”

Crohn’s and Colitis Foundation of America

Statement on Crohn’s Disease 2009
It is here that researchers in the field are left with the results of the high throughput genetic arrays and epidemiologic associations between a disease and a gene. It is now the goal of both basic science and translational researchers to uncover the possible mechanisms that underly this mutational phenotype and possible circuits that explain the diverse array of malfunctions we see within the gut of a patient with Crohn’s Disease.
EXAMINATION OF A CIRCUIT
PRIMARY RESEARCH OF THE NOD2 DEFECT IN THE CX3CRI⁺ LPDC
AND IT’S EFFECT ON THE ILEAL MUCOSAL NICHE
Mutations in the Human NOD2 Gene Are Associated with an Altered CX3CR1+ Intestinal Lamina Propria Dendritic Cell

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INTRODUCTION

The nuclear oligomerization binding domain-2 (NOD2) gene plays an essential role in human intestinal health by coding for an intra-cytoplasmic muramyl-dipeptide (MDP) receptor found in enterocytes, Paneth and antigen presenting cells (APCs) (1). Within APCs NOD2 is capable of bacterial sensing and initiation of an immuno-modulatory response (2, 3). Several NOD2 single nucleotide polymorphisms (SNP 8, R702W; SNP 12, G908R; and SNP 13, L1007fs) are associated with intestinal pathology in man, of which Crohn’s disease (CD) is the most prevalent (4, 5). The NOD2 receptor binds MDP within a phagolysosome activating a pro-inflammatory/anti-apoptotic response via NFκB release from IκB at the cytoplasmic membrane (6-8). In addition, further associations with NOD2, the autophagy pathway and alterations in APC cytoskeletal elements have recently been discovered. This suggests a complex role for NOD2 in processing bacterial load, leukocyte mobilization and ultimately immunologic homeostasis within the bowel (9, 10).

To better understand the contributions of the NOD2 expressing epithelium and NOD2 cells of hematopoietic origin in man we have begun studies in patients who have received an intestinal allograft. There are several reasons this “model” provides a unique window on the human immune system (11). First, standard of care requires the prospective monitoring of tissue samples from time of transplant for months thereafter by way of serial intestinal biopsies where the mucosa is sampled for surveillance of infection, inflammation and rejection. This permits the longitudinal study of both the epithelial and dendritic cells of the intestine from an individual living small intestine in the absence of disease, since the forceps of the device can capture both
the epithelial and lamina propria compartment. In addition, changes in the lumenal contents of the small intestine, including commensal microbes can be followed by serial collection of effluent (pre-developed stool) directly from inside the ileostomy (12). Second, about 30% of our recipients harbor CD associated NOD2 polymorphisms, while donor organs exhibit the genotype frequency expected for a randomly sampled population (<3%)(13). This permits us to evaluate the effects that occur at the interface between NOD2 mutant lamina propria and NOD2 wild type epithelium in a living human chimeric organism. Third and finally, within 3 months following engraftment, the allograft becomes truly chimeric in that the epithelium (epithelial compartment) remains of donor genotype, secondary to Lgr5+ stem cell carry over, while the NOD2 expressing cells of hematopoietic origin that populate the graft, such as the dendritic cells and the macrophage, are of recipient origin (14). It is at this 90 day indicator mark that the vast majority of donor bone marrow derived immune cells have been removed from the lamina propria compartment, while the recipient’s have migrated from the bone marrow and taken their place.

As we previously reported, when an otherwise “healthy” donor intestine (from a NOD2 wild type donor) is transplanted into a recipient who carries a NOD2 mutant genotype, the allograft is 100-fold more likely to develop immunologic graft loss due to a chronic inflammatory state secondary to an altered milieu of cytotoxic cytokines concentrating in regions of the ileum (Figure 1 and Figure 2) (13). Curiously, as is also seen in Crohn’s disease, the expression of the Paneth cell (PC) defensin αHD5 was also significantly depressed, suggesting that a failure of local antimicrobial defense might contribute to this inflammatory condition. These findings suggest that there is more to the initiating inflammatory event than discrete
independent immune abnormalities and that there may possibly be a circuit or web of communication being dictated from some central cellular entity capable of hierarchical oversight of the crypt-villus niche, dictating response pathways to intestinal organisms and inflammatory modulators.

Figure 1. Expression of intestinal antimicrobial polypeptide (AMP) genes in tissue specimens from allografts in recipients with or without NOD2 Crohn’s disease (CD)-associated polymorphisms. Recipients represent those in the transplant population that had not as yet experienced a rejection episode and had normal intestinal histology. All donor organs were
from NOD2 wild-type donors (see text). Total RNA was isolated and analysed as described in the Methods section. The relative level (normalised to glyceraldehyde phosphate dehydrogenase) of target mRNA was determined by quantitative reverse transcriptase-PCR. Data are expressed as relative levels of each mRNA, as described in the figure, with the comparator assigned a value of 1.0. (A) Organs sampled prior to transplantation; tissue levels of specific AMPs from allografts implanted into recipients with a NOD2 wild-type genotype (W, white bars) (N=6) are compared with those implanted into recipients with NOD2 mutant genotypes (M, grey bars) (N=4); (B) AMP mRNA recovered from allografts, 3–4 weeks after transplantation into recipients with wild-type NOD2 loci; white bars, tissue samples taken from the donor bowel prior to transplantation; grey bars, biopsies taken from the same bowel 3–4 weeks after implantation. (C) as B, but allografts were transplanted into recipients with NOD2 CD-associated single nucleotide polymorphisms. (D–G) Western blot analyses of AMPs extracted from allograft biopsies taken at the same time points as in B and C, and performed as described in the Methods section. Amounts of protein extract were normalised based on β-actin (β-A) present. Quantitative densitometry of the photographic images for each peptide is presented, along with one representative blot image (*p<0.05; **p<0.01). (H, I) Representative light microscopic images of Paneth cells taken from biopsy specimens corresponding to samples studied in B and C, respectively (phloxine–tartrazine). Figure taken in complete form from previous published work *Gut. 2008 Mar;57(3):323-30. Epub 2007 Oct 26.*

![Kaplan–Meier curves of rejection and graft loss after transplantation associated with the recipient’s NOD2 genotype.](image)

(A) Time to first episode of severe rejection; (B) time to loss of the allograft. One NOD2 wild-type recipient was transplanted twice and was scored as two transplants. Significance was determined by a log-rank test. Figure taken in complete form from previous published work *Gut. 2008 Mar;57(3):323-30. Epub 2007 Oct 26.*
In this report we extend our previous observation by examining the chain of events that unfold in the human intestine, as it undergoes repopulation with NOD2 mutant hematopoietic cells, prior to any histologic inflammation in order to determine whether the cell responsible for sentinel oversight of the crypt-villus niche is indeed from the bone marrow and not necessarily of epithelial origin. It is with this hypothesis, that we have focused our attention on the CX3CR1\(^+\) LPDC, a remarkable cell that has recently been recognized to serve a sentinel function within the small intestine by residing in the lamina propria compartment but being able to sense lumenal activity and subsequently augment epithelial response mechanisms (15-17). We show here that the CX3CR1\(^+\) LPDCs bearing CD associated mutations in the NOD2 gene are unable to extend their characteristic trans-epithelial dendrites (TEDS) despite an abnormally heavy bacterial presence on the epithelial surface and a corresponding robust CX3CL1 stimulation; furthermore, they fail to express many of the gene products at the levels observed for the corresponding NOD2 wild type LPDC, including cytokines involved in immune regulation as well as the growth factor Wnt5a. Microbes become adherent to the wall of the intestine populated by these NOD2 mutant LPDCs before any evidence of intramural inflammation or physical disruption of the barrier. The expression and processing of the Paneth cell defensin \(\alpha\)HD5 is markedly impaired. These observations, based on longitudinal studies in the human small intestine identify the importance of NOD2 in the biology of the CX3CR1\(^+\) LPDC and suggest the existence of communication pathways between itself and the epithelium, which could be disrupted in Crohn’s disease.

With the knowledge that the CX3CR1\(^+\) LPDC acts as a potential sentinel within the terminal ileum we examined whether CD associated SNPs within the cell under a chimeric
system would indeed alter the mucosal niche toward an inflammatory state. To do this, we analyzed the functional physical phenotype and expression profile of the LPDC, in addition to its ability to communicate with the donor derived Paneth cell, in a chimeric setting where the NOD2 mutant recipient bone marrow derived LPDC could interface with the donor NOD2 wild type derived epithelium.

RESULTS

Question 1: Do SPNS within the NOD2 gene alter the apparent phenotype of the CX3CR1+ LPDC within the repopulated intestinal allograft?

To characterize the physical phenotypic consequence of the NOD2 mutation on the CX3CR1+ LPDC and epithelium, prospective surgical specimens and biopsies of the intestinal mucosa were obtained from previously NOD2 genotyped patients allowing us to study the CX3CR1+ LPDC in chimeric patients within the two compartment interface. These prospective biopsies were collected using standard clinical practice by a licensed faculty gastroenterologist at Georgetown University Medical Center under approved IRB conditions. These specimen were collected at approximately 0, 30, 60, 90 and 120 days post intestinal allograft transplant, at which time, recipient blood vessels were anastomosed to the bowel, permitting the delivery of recipient blood to donor tissues.

From these tissues, two groups were studied:
(1) 19 recipients with NOD2 polymorphisms engrafted with an intestine from a NOD2 wild type donor

(2) 11 recipients with NOD2 wild type genotype engrafted with a bowel from a wild type donor.

Of the mutant recipients 13 of 19 were heterozygous for one of the common NOD2 polymorphisms, while 6 of the 19 were homozygous at one or more loci within the NOD2 allele.

Determination of the NOD2 allelic mutational SNPs among the study cohort was determined using a Taq-man directed RT-PCR protocol which required establishing the baseline genotype in both donor and recipient pre-transplant tissues. These tissues and/or blood were used to extract DNA and to evaluate the three Crohn’s Disease associated single nucleotide polymorphisms in the NOD2/CARD15 gene. These SNPs are represented as:

(1) SNP 8 which is a missense mutation located at R702W and documented at NCBI as “SNP ID: rs2066844”

(2) SNP 12 which is also a missense mutation located at G908R and documented at NCBI as “SNP ID: rs2066845”

(3) and finally SNP 13, a specific frameshift C-mutation which results in protein truncation located at 1007fs 3020insC and documented at NCBI as “SNP ID: rs2066847”

RT-PCR probes for these three CD SNPs were supplied by Applied Biosystems and contained both forward and reverse primers and VIC/FAM fluorophor indicators. With the supplied PCR substrates, 10ng of isolated genomic DNA was added to and mixed with 12.5 ml of the Applied Biosystems TaqMan Universal PCR Master Mix No AmpErase UNG. The
amplification assay was performed on both the ABI 7500 Fast Real Time PCR and the ABI 7900 Fast Real Time PCR Device.

From these results, donor and recipients were assigned either wild type, heterozygous or homozygous NOD2 mutational status at the indicated SNP location. Each recipient in the study was then documented with their apparent correlative primary pathology, age, gender and chimeric status as indicated in the (Table 1.) These patients define the cohort examined over the study and those biopsies collected from each of the indicated patients was stored in the Transplant Institute’s IRB approved tissue bank for further analysis.

Over the above outlined period of biopsy collection, some samples of the tissues were evaluated for recipient LPDC repopulation of the donor allograft in order to track movement of recipient bone marrow derived dendritic cells into the lamina propria compartment of the donor. This observational tracking of the chimeric status required the precise isolation of the CX3CR1$^+$ LPDC itself from the biopsy tissue, a process which will be discussed later in detail. From these dendritic cells, predetermined HLA allele variations were compared, which were previously acquired from the Clinical Transplant HLA lab in order to determine the relative percentages of donor and recipient CX3CR1$^+$ LPDCs in the tissue over time. In short, the HLA allelic variations that exist between donor and recipient cells can be used to determine the relative quantity of cells when drawn from a chimeric heterogeneous tissue. From this data it was discovered that the recipient DC repopulates the donor graft and nearly defines the vast majority ~100% of the total CX3CR1$^+$ LPDC population at approximately 90 days post engraftment (Figure 3.)
Table 1. Intestinal Transplant Patient Study Cohort. The population of allograft recipients described in this report and their correlative demographics and primary disease pathology leading to the requirement of intestinal transplantation. Donor and Recipient baseline genotypes are indicated which relate to continued epithelial expression of the donor genotype secondary to the Lgr5\(^+\) stem cell maintained in the crypt, while the repopulating bone marrow derived cells maintain the recipient genotype. The two individuals who received allografts from donors with NOD2 mutant genotypes are defined as reverse mutants.
Figure 3. Determination of recipient CX3CR1⁺ LPDC repopulation kinetics. DNA was isolated from FACS purified CX3CR1⁺ LPDC as described in Methods. HLA alleles associated with the recipient and donor were determined prior to transplant through clinical cross-matching. The concentration of each of these alleles in the CX3CR1⁺ LPDC DNA sample was separately
assayed using two appropriate allele primer sets specific for the donor and recipient. In the representative example shown, LPDCs were isolated at 90 days and 115 days post intestinal engraftment. The relative concentrations of two donor-specific alleles (DRB1*0801, DRB1*04) and two recipient-specific alleles (DRB1*0302, DRB1*07) were determined in the DNA samples. White arrows indicate the correct base pair PCR product or its expected position (indicated beside each lane) corresponding to the particular HLA allele of both donor and recipient based on the prior analysis of DNA from a blood specimen. DNA base pair ladders are indicated at the right of each 2% agarose gel. PCR products were visualized with ethidium bromide under UV light. In this figure, the donor DRB1 *0801 (170 bp) and recipient DRB1 *0302 (175bp) bands generated in the specified DNA mixtures are shown. A comparable analysis was conducted for the donor-recipient pair DRB1*04-DRB1*07. The mixtures were generated by from the specified proportions of donor and recipient DNA. **(Lanes 1, 2, 90 days; Lanes 1, 2 115 days):** Donor DRB1 *0801 (170 bp) and Recipient DRB1 *0302 (175bp) bands. Based on the relative intensities of these bands with the standards, the CX3CR1⁺ LPDC preparation obtained at 90 days post-transplant was estimated to contain 90% recipient cells, while the preparation obtained at 115 days, was closer to 100% recipient. **(Lanes 3, 4, 90 days; Lanes 3, 4, 115 days).** Similar results were obtained with the second set of alleles, the LPDC estimated to contain about 90% recipient cells at 90 days post-transplant and close to 100% at 115 days.

Understanding the dynamics of CX3CR1⁺ LPDC repopulation of the graft is vital to question being asked in this section in that we must first determine the chimeric status of the patient and then be able to determine which LPDC (donor or recipient) is exhibiting the phenotypic abnormality within the chimeric tissues. In order to ensure that repopulation by the recipient bone marrow derived CX3CR1⁺ LPDC had taken place, biopsies were harvested from allografts at various times after initial engraftment; the CX3CR1⁺ LPDC was isolated using fluorescent activated cell sorting (FACS) gated on three published identifying markers (CX3CR1, CD11b, CD11c) that have been used to characterize this cell population in the mouse (15, 16). The isolated LPDC population was then evaluated for expression of donor and recipient specific HLA allelic markers, characterized during pre-transplant cross-matching.
90 days post-transplant the lamina propria compartment contained >90% recipient CX3CR1+ LPDCs and by day 115, approximately 100% recipient derived (Figure 3).

In short, the concentration of recipient and donor DNA was evaluated using standard SSP-PCR HLA typing methods according to the manufacturer’s protocol. Amplified DNA was visualized on a 2% agarose gel stained with ethidium bromide. Based on recipient and donor pre-transplant HLA typing, informative primer pairs were chosen from low resolution HLA-A or HLA-DRDQ SSP Unitray kits (Invitrogen; Brown Deer, WI). At least one donor-specific SSP primer pair and one recipient-specific primer pair was included along with a primer pair expected to be negative with both the donor and recipient in order to control for non-specific amplification. Dilutions of amplified donor and recipient DNA were analyzed to evaluate the relative amounts of DNA present and compared to a fixed known scale of DNA concentrations to determine percentages as described in the figure caption.

From Figure 3, we can clearly see that the recipient bands increase in intensity from day 90 to day 115, while the donor bands disappear over that time period. This suggests that there is nearly complete replacement of the donor CX3CR1+ LPDC, which were brought over in the initial allograft transplantation event, by the bone marrow derived recipient CX3CR1+ LPDC.

Now after understanding the dynamics of the CX3CR1+ LPDC repopulation of the small intestinal lamina propria compartment by the CX3CR1+ LPDC, those with a NOD2 mutation take on an altered microscopic appearance compared to the NOD2 wild type CX3CR1+ LPDC (Figure 4). We have noted this peculiar morphology in all 19 mutant recipients studied. Three random representative examples of LPDCs heterozygous for one of the common NOD2 CD associated polymorphisms are presented here (Figure 4 i-l). These CX3CR1+ LPDCs do not
extend the characteristic TEDs as demonstrated by FITC fluorescent labeling of CX3CR1 and do not organize their F-actin cytoskeleton in an attempt to “sense” luminal microbial organisms. In contrast, the NOD2 wild type CX3CR1⁺ LPDC does extend the characteristic TEDs observed throughout the literature and appears to organize the proper intracellular F-actin support structure (Figure 4 a-f). The expression of both CX3CR1 protein and mRNA within the CX3CR1⁺ LPDC were found to be higher in the NOD2 mutant than the wild type DC demonstrating that the observed morphological abnormality is not due to a deficiency in the CX3CR1 receptor at either mRNA or protein levels (Figure 5).

Figure 4. Phenotypes of NOD2 wild type and NOD2 mutant CX3CR1⁺ LPDCs from the human ileum. Representative microscopic images from allograft biopsies taken from 2 NOD2 wild type (a-f) and 3 NOD2 mutant recipients (g-j) R702W/wt; (k) G908R/wt; (l) L1007fs/wt, each at about 90 days post-transplant. Tissues were processed and prepared for immunofluorescent analysis as described in Methods. Sub panels (a,g) DNA(DAPI, blue); (b,h) CX3CR1 (FITC, green); (c,i) F-actin (Cy3, red); (d,e,f,j,k,l) merged color images (Scale bar, 10 microns). Area outlined in the white rectangle in (d) is shown in (e). White arrows denote the trans-epithelial dendrite extensions that characterize these LPDCs of the terminal ileum.
Figure 5. **CX3CR1 Protein and mRNA expression by the CX3CR1+ LPDC.** (a) Average relative FITC/green channel fluorescent intensity correlating to CX3CR1 protein presence as it relates to the NOD2 status of the CX3CR1+ LPDC in the previous images. Relative standardized mean of FITC fluorescence in NOD2 WT 0.46 SD±0.04 vs. NOD2MT 1.0 SD±0.2 (b.) RT-PCR assay of relative CX3CR1 mRNA expression of FACS isolated CX3CR1+ LPDCs as it relates to the NOD2 status of the cell. Relative standardized mean of RT-PCR SYBR Green mRNA in NOD2 WT 0.57 SD±0.03 vs. NOD2MT 1.0 SD±0.12. Graphs represent averaged relative expression of transplant cohort NOD2 WT n=11, NOD2 MT=19. (*) Represents a p-value of <0.5.
Question 2: Are Single Nucleotide Polymorphisms in the NOD2 Gene Associated with Altered Expressions Patterns in the CX3CR1+ LPDC?

With understanding that the donor NOD2 wild type CX3CR1+ LPDC is replaced with the recipient NOD2 mutant CX3CR1+ LPDC and that those cells appear to not extend the characteristic TED which defines them, we conducted a focused transcriptional analysis on all mutant and wild type LPDC samples in our chimeric transplant study population. We used FACS isolated dendritic cells from biopsy samples by targeting three published LPDC identifying markers (CX3CR1, CD11b, CD11c) (Figure 6 A and B).

The unique process of isolation involves tissue collected prior to donor small bowel implantation and during follow-up protocol endoscopic biopsy. These biopsies and tissue specimen were placed directly into cold (+4°C) HBSS/phenol red solution (Cellgro) in the operating room and/or endoscopy suite before processing in the laboratory. These specimen were then brought directly to the GUMC Transplant Laboratory where they were immediately washed for 10 minutes on a slow rocker with PBS (GIBCO) to remove particulate matter. Next, to remove the mucus layer, the samples were rocked for 10 minutes in 1% DTT (Fluka Intl) PBS solution. The epithelial layer was separated from the lamina propria by means of rocking the biopsies in 30mM EDTA-PBS solution (Boston BioProducts) for 15 minutes. Under these conditions the epithelium separates as a cellular sheet leaving the lamina propria as an amorphous fragment of tissue that can be easily isolated away from the sub-epithelial tissues. Both tissues were physically transferred with micro-tipped forceps onto a 40 µm nylon filter and washed to remove debris, and then transferred to PBS. Here, the epithelium was utilized for
subsequent analyses, including the ligand challenge assays described later. The lamina propria was chemically de-aggregated into individual cells by treatment with Collagenase-IV, DNase-I and 5% fetal calf serum (Stem Cell Technologies) for 90-120 minutes while rocking at 37°C. Digested tissue was then rinsed with PBS and filtered through a 40 μm nylon membrane (BD Falcon). The filtered cells were spun down into a pellet at 1850 rpm for 5 minutes in a microtube centrifuge (Eppendorf). The pellet was washed with PBS and then blocked with 10% normal donkey serum (Jackson Immunology) for 15 minutes to reduce non-specific binding interactions. After blocking, the cells were washed with PBS and spun down into a soft pellet. After brief vortex re-suspension, the free floating cells were labeled with fluorescent antibodies directed at three cell surface epitopes on the dendritic cell, based on comparable studies in the mouse: CX3CR1-FITC (MBL), CD11b-PE (Abcam), CD11c-PE/Cy7 (ebioscience) for 60 minutes at +4°C. Cells were washed twice with PBS to remove excess antibody and re-suspended in FACS buffer (1% BSA, 0.01% sodium azide) (Sigma) and filtered for sorting at the Georgetown FACS Core Facility using a BD FACSARia III (BD Biosciences). After the cells were sorted via flow cytometry, isolated LPDCs were spun down and stored in a RLT/BME solution at -80°C for further RNA/DNA/Protein isolation and clean up (QIAGEN, All Prep DNA, RNA, Protein isolation kit).

From these isolated cells, determination of mRNA expression was completed using custom focused designed RT-PCR arrays. In brief, mRNA was isolated from FACS isolated cells using DNA/RNA/Protein All-Prep kit (Qiagen) followed with RNA cleanup and concentration using RNeasy MiniElute and Cleanup kit (Qiagen). RNA was quantified using a NanoDrop (Fisher Scientific). RNA was converted to cDNA using SABiosciences cDNA first strand kit
according to protocol. The cDNA was then added to MQH$_2$O and SABiosciences SYBR Green/ROX master mix in a 50ml tube (Corning) and then placed in a sterile 12 tip multi-channel pipette trough (VWR). Using the multi-channel pipette, 25 µl/well of material (100ng total RNA) was then added to the custom RT2-PCR pathway array 96 well plate (SABiosciences). The RT-PCR assay was conducted using the protocol provided by the SABioscience plate in ABI 7500 Fast System unit. Abundance is expressed in generalized units relative to in plate controls and the GAPDH housekeeping gene. The data analysis was conducted using the SABiosciences online RT2-PCR Pathway Array Software ®, which can be found at www.sabiosciences.com.

Having now developed the ability to isolate intact CX3CR1$^+$ LPDCs, we found that several highly significant differences were evident between the transcriptomes of the mutant LPDCs (as a group) and the NOD2 wild type LPDC (Figure 6 C). The NOD2 mutant CX3CR1$^+$ LPDC did not express either TLR1 or TLR2 as measured within the dynamic range of our RT-PCR array. The expression of ICAM 1 and 2, MIF, inhibin beta A subunit, and midkine were also significantly depressed when compared with the wild type DC population (Figure 6 C). Among the genes more highly expressed in the NOD2 mutant LPDC were those encoding the proteins CX3CR1, TNFα, IL-8 and IL-2 and IFNγ. These findings suggest parallel expression profiles to those found in published literature concerning the up regulation of inflammatory cytokines in CD patient tissues.

In addition to alterations in the mRNA expression profile there were also differences in cytokine protein expression between the NOD2 wildtype and mutant dendritic cells as well, which was determined by enzyme linked immunosorbant assays using a “Multi-analyte
inflammatory cytokine ELISA kit ®” (SABiosciences). From the study it appears that a mutation in the NOD2 allele within the CX3CR1⁺ LPDC predisposes or biases the cell towards inflammation as determined by the cell’s cytokine protein expression profile (Figure 6 D). This inflammatory profile pattern appears evident in all three of the Crohn’s disease associated SNPs. In particular, at the protein level, the abundance of IFNγ and TNFα appeared to be significantly elevated, even at times where the mucosa was deemed “normal” by a clinical pathologist. Infact, as can be seen from the averaged multi-analyte cytokine data in Figure 5, when comparing IFNγ and TNFα levels, the chimeric patients carrying the NOD2 SNPs within the LPDC seem to exhibit 7 to 8 fold the relative value units than the patients who possess NOD2 wild type dendritic cells. These results suggest that a similar cytokine profile has evolved within these patients as exists within the “typical” Crohn’s disease patient. This typical molecular phenotype can be defined as a type of self destructive cytokine augmentation effect where IFNγ, also known as a “macrophage activing factor,” promotes inflammatory leukocyte infiltration of the tissue, which subsequently results in chronic inflammatory changes and destruction of the bowel wall.

These findings would suggest the same deleterious molecular alterations occur within the LPDC, which prevent the dendritic cell from not only properly expressing certain members of the TLR, ICAM and MIF families but also induce a misdirected inflammatory response by augmenting the incorrect cytokine profile leading to inappropriate cellular destruction when in fact invading micro-organisms are truly the concern.
Figure 6. mRNA and protein expression profiles of NOD2 wild type and NOD2 mutant CX3CR1+ LPDCs isolated from the human ileum. Isolation of CX3CR1+ LPDCs by fluorescence activated cell sorting. Procedures are described in SI Methods. A. Gating on CX3CR1 (FITC), CD11b (PE) B. Secondary gating on CD11c (PE/Cy7) in addition to forward and side scatter. Black arrows indicate the selected populations. C,D. Expression analyses of FACS isolated CX3CR1+ LPDCs. Cells were processed for PCR (C) or ELISA (D) studies as described in Methods. C. Focused RTPCR expression profile comparing the isolated CX3CR1+ LPDC as it relates to its NOD2 wild type and mutant genotypes. (n=30, 11 NOD2 wild type, 19 NOD2 mutant). D. Focused ELISA analysis of cytokine expression profile of the FACS isolated CX3CR1+ LPDC. Cytokines across 3 heterozygotic NOD2 single nucleotide polymorphisms R702W (n=8), G908R (n=7), L1007fs (n=4), and wild type (n=6) are represented as averaged relative protein expression values as described in Methods. IFN-γ NOD2 MT mean 0.33 SD±0.05 NOD2 WT mean 0.018 SD±0.01 relative units; TNF-α NOD2 MT mean 0.36 SD±0.07 NOD2 WT mean 0.011 SD±0.009 relative units. Abbreviations Wild type (WT), Mutant (MT), Interleukin (IL), Interferon gamma (IFN-γ), Tumor necrosis factor alpha (TNF-α), Granulocyte-macrophage colony stimulating factor (GM-CSF). NS, not significant. (*) indicates P value <0.05.
Question 3: Does the NOD2 SNP Associated Blockade of the CX3CR1⁺ LPDC TED Extension Permit the Accumulation of Bacteria on the Mucosal Barrier of the Small Bowel?

The phenotypic and expression abnormalities seen within the NOD2 mutant CX3CR1⁺ LPDC, correlate with what appears to be a loss of function by this cell in dealing with gut microorganisms. In order to determine microbial adherence and invasion, fluorescent in situ hybridization methods developed by Swidsinski et al. were employed. We utilized the “universal” bacterial nucleic acid probe EUB338, which targets the 16S rRNA of bacteria (18, 19). Briefly, these studies involved collection of biopsies at protocol specified times and involved the immediate fixation of the biopsy in a suitable medium that preserved microbial epithelial attachment (Carnoy’s solution) and allowed for characterization of the presence of microbes by fluorescent in situ hybridization using a “universal” bacterial nucleic acid probe targeting bacterial 16S rRNA conjugated to the chromophore Cy3 for microscopic visualization. In addition, 4’, 6-diamidino-2-phenylindole (DAPI) was used to visualize DNA content of the adherent microbes, in order to verify the existence of a true organism. These biopsy specimen samples were collected on pre-genotyped mutant and wild-type NOD2 small bowel transplant patients at the time of primary allograft transplantation and subsequent follow-up biopsies. These specimen were immediately transferred, after acquisition, to the Carnoy’s fixative for 6 hours at +4°C. Tissues were then paraffin embedded and sectioned at 3μm (12 sections total). H&E, periodic acid Schiff and Gram stains were applied to six of the twelve sections, while the remaining six were prepared for 16S rRNA fluorescent hybridization with the universal bacterial probe 5’Cy3-EUB338:
[*Cy3*5'- GCT GCC TCC CGT AGG AGT -3'] (MWG Biotech)

Upon removal of the paraffin and specimen section re-hydration using a standard xylene/ethanol protocol, the tissue was warmed for improved slide adherence, and encircled with a Pap-pen ® (Invitrogen) to prevent hybridization buffer evaporation. Treatment with mutanolysin allowed better probe penetration of Gram positive organisms. FISH hybridization was conducted in a humidified 37°C incubator (Fisher Scientific) for 90 minutes at probe concentration of 10 µg/ml in the stringency confirmed hybridization buffer [0.9 M NaCl, 5% Formamide, 10% SDS 1M Tris-HCl (pH 7.2)]. Following a post hybridization buffer wash, a non-EUB338 probe was applied to remove non-specific background of 5’Cy3-EUB338. After removal of excess EUB338, DAPI was used to identify all nucleated cells within the section. The section was washed and sealed with a cover slip, following application of Pro-Long Gold ® anti-fade reagent (Invitrogen) and evaluated using epi-fluorescence and confocal microscopy.

It is clear from these protocols and our studies that adherence to the epithelium by microbes occurs in allografts that contain a NOD2 defective LPDC (Figure 7 A c-e) while the epithelium from allografts carrying NOD2 wild type DCs appear relatively free of adherent bacteria (Figure 7 A a,b; 9B). Immunohistochemical analyses targeting bacterial endotoxin within the intestinal mucosa, an indication of the invasion of epithelial barrier by bacteria, was seen only in allograft specimens associated with NOD2 mutant LPDCs (Figure 7 C e-h), and not wild type LPDCs (Figure 7 C a-d). Further evaluation using Confocal Z-plane analysis of the 16S rRNA hybridized specimen indicated that the bacteria are not only adherent to the mucosal
wall, as initially thought, but also found to have invaded the intracellular compartment of the cell (Figure 8).

Beyond bacterial adhesion and invasion occurring within the chimeric mucosa containing the NOD2 mutant CX3CR1+ LPDC, we also see significantly increased CX3CL1 expression by immunofluorescence (Figure 6E d-f), as compared with specimens carrying NOD2 wild type CX3CR1+ LPDCs (Figure 7 E a-c) and confirmed by the semi-quantitative analysis of protein by immunoblot (Figure 7 F, G), by the abundance of mRNA by PCR (Figure 7 H), and by ELISA of protein extracted from epithelium (Figure 9). This recently discovered fourth family of chemokines (CX3C) remains the only known ligand to CX3CR1 and is primarily produced by the enterocyte. Increased levels within the bowel indicate an inflammatory “stress” response to microbial presence and should direct CX3CR1+ LPDC TED extension through proper F-actin cytoskeletal support (16, 20) a response we do not observe with the mutant NOD2 CX3CR1+ LPDC. These results, with reference to the earlier section suggest that the NOD2 mutant LPDC not only express more CX3CR1 mRNA and protein but also exist in a focal region where there is also more CX3CL1 protein ligand produced.

These resultant findings would suggest that there is no lack of substrate for the CX3CR1/CX3CL1 TED directed extension axis but rather an extra-axial malfunction occurring within the LPDC which then subsequently prevents the fractalkine dendrite extensions from occurring despite significantly increased bacterial load both adherent to and invading within the intestinal mucosa. Having both increased expression of the CX3CL1 ligand peptide via increased epithelial stress activation and the abundant presence of micro-organisms at the intestinal wall, one would expect the dendritic cell to follow normal suit and orient its TED polarity toward the
enterocyte/lumen interface, which is clearly not occurring within the defective NOD2 mutant cells. These findings suggest the need to evaluate not only the direct defect that exists within the LPDC but possible abnormalities in the dendritic cells communication within the epithelium’s anti-microbial producing entity, the Paneth cell.

Figure 7. Microbial adherence and invasion of the intestinal wall as it relates to the NOD2 genotype of CX3CR1+ LPDC. A. Bacterial adherence. Representative biopsies from (a, b) NOD2 wild type recipients and (c-e) heterozygotic NOD2 mutant specimens. Bacteria, which appear as the bright yellow granular material, were visualized (FISH) as described in Methods. Each panel represents a representative image from a different patient. Epithelium (closed arrows). Lamina propria (open arrows). B. Relative averaged fluorescent intensity of images in panels A (a-e). C. Intraepithelial LPS. Biopsies from NOD2 wild type (a-d) and heterozygotic NOD2 mutant specimens (e-h) (each from a different patient) were immunostained for LPS as
described in Methods. **D.** Relative averaged red channel intensity of images in C. **E.** Expression of CX3CL1 in a representative biopsy from a NOD2 wild type (sub panels a-c) and a heterozygotic NOD2 mutant (sub panels d-f) patient. Tissue was prepared for immunofluorescent visualization as described in Methods. **Ea, Ed.** DNA (DAPI, blue); **Eb, Ec.** CX3CL1 (Cy3, red); **Ee, Ef.** Merged images. **F.** CX3CL1 detected by western immunoblotting, as described in Methods. CX3CL1 (M) and CX3CL1(S) indicate the larger mucin stalk bound membrane form and small soluble form respectively. Bands associated with the NOD2 mutant sample are barely visible at the exposure presented. **G.** Densitometric analysis of individual CX3CL1 blots (M form) run on all 30 patients, standardized relative to the intensity of the GAPDH signal. **H.** Relative CX3CL1 mRNA abundance in biopsy samples as analyzed by RTPCR, conducted on all patient biopsies, as described in Methods. (n =30, 11 NOD2 wild type, 19 NOD2 mutants). (Scale bar, 10 microns). NS, not significant. (*) indicates P value <0.05.

**Figure 8.** Intraepithelial bacteria in allografts harboring LPDCs with a NOD2 mutant genotype. Bacteria were visualized in the biopsy specimen by FISH as described in Methods and examined microscopically under differential interference contrast (DIC). DIC/Cy3 merged confocal z-plane images. Z-plane image acquisition was taken at ½ specimen depth or 1.5 µm below the cover slip in order to validate an internal Cy3 signal location within the tissue. Specimens from two different patients are presented. (Scale bar, 10 microns)
Figure 9. CX3CL1 fractalkine protein was measured via a customized 96-well SABioscience Mono-analyte ELISA array. Protein was extracted from isolated ileal epithelium as described in previous methods. (Top) Comparison of CX3CL1 epithelium protein expression from allografts containing the indicated NOD2 WT and NOD2 MT dendritic cells within the lamina propria compartment. CX3CL1 protein expression for NOD2 WT Mean 0.14 SD±0.03 NOD2 MT Mean 0.55 SD±0.04 (Bottom) Three individuals heterozygotic for each of the three NOD2 polymorphisms were compared against three wild type recipients in each assay. Samples were run in triplicate and quantified as noted above. (n=18; 3 R702W; 3 G908 R; 3 L1007fs; 9 wild type). (*) indicates a p-value <0.05
Question 4: Does the NOD2 SNP Alter Expression of the Inflammatory Ligand Wnt5a within the CX3CR1+ LPDC?

Recent interest in the Wnt5a ligand acting as an inflammatory signaling molecule in macrophages and dendritic cells when stimulated by microbes, led us to question whether Wnt5a ligand expression would differ between the NOD2 wild type and mutant CX3CR1+ LPDC (21, 22). We initially examined immuno-fluorescently stained tissue specimens microscopically for expression of Wnt5a using tissues that were placed in 10% normal buffered formalin (NBF), paraffin embedded and sectioned to 3μm by a GUMC core facility (Histopathology Tissue Shared Resources). Sample processing and staining were conducted following the general immune-fluorescent paraffin staining protocol provided with the antibody [IF-PARAFFIN PROTOCOL (IF-PP)] (Abcam). The basic protocol of labeling involves deparaffinization and rehydration with 100% Xylene for 8 minutes (3x), 100% Ethanol 3 minutes (2x), 95% Ethanol 3 minutes (2x), 50% Ethanol 3 minutes (1x) and finally MQH2O for 30 seconds. Antigen retrieval was performed with DAKO Antigen Retrieval Solution ®. Tissue sections were immersed into a pre-heated 65°C working target antigen retrieval solution and then incubated for 45 minutes at 97°C in a water bath. DAKO Blocking Solution ® was then applied to the sections for 30 minutes at room temperature in a moist chamber. The blocking solution was “tapped off” the slides and the primary antibody was applied using the DAKO developed Antibody Dilution Solution ®. The antibody (1:50) in the antibody dilution buffer was applied to the tissue sections to create a dome over the specimen. The incubation was maintained at room temperature for 1 hour in a moist chamber without exposure to light. The tissue was then rinsed three times with
PBS (5 minutes per washing) and the secondary conjugate antibody (1:250) applied using the same antibody dilution buffer and allowed to sit for 30 minutes at room temperature. The slide was then washed as previously described. DAPI (Sigma) was applied for 10 minutes followed by a 1 minute rinse in PBS. The slides were air dried, then covered with a film of ProLong Gold ® and sealed with a glass cover slip. Slides were maintained at +4°C overnight before microscopic examination. Imaging was performed on both a Zeiss Axiophot2 epi-fluorescence microscope and Olympus Fluoview confocal microscope. The relative fluorescent and chromogenic intensities were acquired using ImageJ and Sigma Gel software programs. Quantitative analyses of the “total fluorescence” of a microscopic image were conducted as follows: Images from both wild type and mutant recipient specimens were compared to one another using the specified color filter. Each image was divided into 15 segments of equal area, and the image brightness was quantitated using an open-source image processing program (Image J). The relative intensities of all images from NOD2 mutant and wild type specimens in a particular study were determined and catalogued. Among the 30 patients in the cohort, each slide set contained duplicates in order to validate proper labeling. Those specimen, where significant differences existed among duplicate slides due to artifact, were removed from the study.

From these labeled specimen, we observed a dramatic deficiency of Wnt5a expression in the NOD2 mutant CX3CR1 + LPDC when evaluating for correlative immuno-fluorescence compared with the wild type cell (Figure 10). The mRNA expression of several members of the Wnt family, in addition to Wnt5a, could be detected by RT-PCR in CX3CR1 + LPDCs when using a focused Wnt pathway axis custom array. From the averaged mRNA expression data,
using SABioscience web based heat mapping software we were able to determine high throughput expression data on the intestinal transplant study cohort. It was found that Wnt5a, was present at approximately 14-fold lower concentrations in NOD2 mutant dendritic cells compared with the wild type counterpart (Figure 10 C, D). This result would suggest that despite a significant bacterial load present at the mucosal barrier and within the tissue, the MDP was not inducing up-regulation of the Wnt5a gene. With clearly different basal levels of Wnt5a existing within the two dendritic cell populations, one would next question whether or not this phenomenon could be manipulated if the cells were challenged in vitro with the MDP ligand.

To determine whether the NOD2 mutant CX3CR1+ LPDC was functionally defective with respect to its capacity to express Wnt5a we FACS isolated CX3CR1+ LPDCs and exposed the cells, in vitro, to the NOD2 ligand, muramyl dipeptide (MDP). A modified technique of Blumenthal et al. was used to challenge isolated CX3CR1+ LPDCs and quantitate the Wnt5a secretory response. Here, LPDCs were purified by FACS. The isolated cells were then placed in 50 µl RPMI 10% FCS (Sigma) 1x Penicillin, Streptomycin, and Glutamine (GIBCO). Challenges were performed in a 37°C, 5% CO2 incubator in 16 well plates (Falcon). 100 µg/ml of MDP ligand was used to challenge these cells (Sigma).

From this study it was determined that exposure of the NOD2 mutant CX3CR1+ LPDC to MDP resulted both in significantly lower secretion of Wnt5a (Figure 9 E) as well as diminished induction of Wnt5a mRNA (Figure 10 F) In fact, the NOD2 wild type dendritic cell not only maintained higher levels of the Wnt5a mRNA and protein at baseline, it also responded to MDP at 5-fold higher values than that which was observed for the NOD2 mutant LPDC. These results would suggest that the defect itself is intrinsic to the dendritic cell and not necessarily the
environment it was harbored in. The inability to up regulate Wnt5a, both within the live allograft and \textit{ex vivo} system, despite accumulation of bacteria at the intestinal wall, suggests that the NOD2 detection of MDP, in some way, triggers Wnt5a release by the DC. Understanding this, a cell with a NOD2 defect would be expected to have altered Wnt5a expression patterns. In addition, even with excessive direct MDP stimulation of isolated dendritic cells, we are still unable to overcome the NOD2 block, implying that this inability is not simply a result of mass action on the pathway. These findings imply that it is the fundamental defect in NOD2, blinding the DC to MDP detection, that correlates with both the molecular inflammatory profile and the clinical appearance we see in Crohn’s Disease.
Figure 10. Wnt5a CX3CR1⁺ LPDC expression as it relates to the NOD2 genotype. A, B. Representative microscopic image of an allograft biopsy from a (A) NOD2 wild type or (B) heterozygotic NOD2 mutant recipient. Specimens were prepared for immuno-fluorescent analyses as described in Methods.  

Aa, Ba. DNA (DAPI, blue); Ab, Bb. Wnt5a (Cy3, red); Ac, Bc. CX3CR1 (FITC, green); Ad, Bd. Merged images. C, D. Expression of Wnt related genes from FACS isolated LPDCs as analyzed by RT PCR in a 96 well plate array. Analyses from individual NOD2 wild type and mutant samples were averaged. C. Heat map of relative expression data shown in D. E. Secretion of Wnt5a from isolated LPDCs in response to various stimuli. LPDCs were isolated by FACS from each of the 30 patients in the study and exposed to the listed stimuli in vitro for indicated times. Concentration of Wnt5a in the fluid was determined by ELISA as described in Methods and expressed in relative concentration units. F. Stimulation of LPDC Wnt 5a mRNA expression by MDP. Relative mRNA abundance was determined by RT PCR. (n =30, 11 NOD2 WT, 19 NOD2 MT). (Scale bar, 10 microns). NS, not significant. (*) indicates P value <0.05.

Question 5: Understanding that NOD2 SNPs alter the LPDC Expression of Wnt5a through Blinding the Cell to MDP, Does Depressed Wnt5a Lead to Lower Levels of the Anti-microbial Peptide Human Defensin 5 Produced by the Paneth Cell?

Epithelial derived Paneth cells of the adult small intestine, in both mouse and man, are regulated by Wnt stimulation (23, 24). Wnt, acting through its receptor, Frizzled (Fzd), stimulates several Paneth cell genes that encode anti-microbial proteins and peptides, including human alpha defensin 5 (αHD5). Stimulation of the Wnt/Fzd circuit results in intra-nuclear translocation of T cell factor-4 (TCF4). This activation of TCF4 subsequently leads to αHD5 gene activation (25, 26). Synthesis of the antimicrobial peptide αHD5, leads to greater granularity of the Paneth cell, which store these peptides as zymogens, for release at a later time. Once these pro-peptides are exocytosed, they are cleaved by trypsin in order to form the mature 3.5 kDa cationic protein, which is capable of forming multimeric structures resembling pores
that act similar to the MAC complex of the complement cascade. These pores punch holes in the bacterial cell wall, destroying the osmotic and electrical gradient (27). Destruction of this osmo-electrical gradient results in the anti-microbial effect which defines Human Defensin 5. Depressed levels of αHD5 have been associated with increased bacterial adhesion to the small intestinal mucosa, gut inflammation, ileitis and finally with the development of the clinical picture of Crohn’s Disease. It is both quantity and quality of this agent that permits it to act as an anti-microbial peptide effectively.

As we had reported previously (13), Paneth cells from allografts populated with NOD2 mutant LPDCs exhibited fewer and smaller granules (Figure 11 A) than observed from specimens populated by NOD2 wild type LPDCs (Figure 11 A a; Figure 11 A c). The numbers of Paneth cells/crypt did not differ between the two groups. By semi-quantitative immunoblot analysis of protein isolated from the tissue specimens, we determined that the allograft epithelium from NOD2 mutant recipients (regardless of genotype) yielded about 20% the amount of αHD5 peptide as compared with the amount of peptide recovered from NOD2 wild type specimens (Figure 11 A e, 11 A c), with a corresponding depression in αHD5 mRNA abundance (Figure 11 A d). Surprisingly, lumenal samples obtained from allografts populated with the NOD2 mutant LPDC contained little mature 3.5 kDa αHD5 peptide. Instead, the majority of this peptide was represented by the unprocessed ~10 kDa “pre-pro-form,” a species not observed in samples from NOD2 wild type recipients (Table 2). A comparable defect in αHD5 processing has previously been reported in luminal samples from patients with ileal Crohn’s disease, who have not undergone intestinal transplantation (28).
Here, among our studies, Human Defensin 5 analysis was conducted on ileal lumenal fluid from the stoma of the graft. These analyses required 1ml of fresh allograft effluent (precursor to stool), free of any fixatives and obtained by endoscopy through the ileal stoma in the morning prior to feeding. This effluent material was then added to 0.5 ml of 60% acetonitrile (Sigma) 1% TFA (Sigma) solution in 4 ml MQH2O (Cellgro). The sample was then vortexed for 20 minutes at room temperature, sonicated and centrifuged for 10 minutes at 14,000 rpm for debris clarification. Supernatant from the clarified effluent material was then transferred into 1.7 ml Eppendorf tubes and centrifuged at 14,000 rpm for an additional 20 minutes for further clarification. These clarified supernatants were then combined in a fresh 15 ml centrifuge tube. 10 ml of -20°C 100% molecular grade ethanol (Sigma) was then added to the supernatant and vortexed briefly. The mixture was then allowed to sit in a -20°C freezer overnight in order for the protein to precipitate out of the aqueous phase. This precipitated mixture was then centrifuged at 14,000 rpm for 20 minutes at +4°C. The majority of the supernatant was then removed leaving ~25 µl of supernatant over the pellet region of the precipitated protein. The pellet was then washed with 95% ethanol. The ethanol wash was then removed and the tube was inverted and allowed to air dry for 15 minutes. The dried pellet was then re-suspended in 100 µl of TBS (Bio-Rad). 100 µl of lamelli loading buffer (Bio-Rad) was then added, mixed briefly and placed onto a 90°C heat block for 10 minutes. Here, the protein containing loading buffer was then added to a 12 well 10-20% Tris- Tricine stack gel and electrophoresis was performed using a Criterion Blotter gel electrophoresis cassette (Bio-Rad). Electrophoresis occurred at 75V for 3 hours, or until proper lane and ladder separation was observed. A PVDF membrane was used for transfer and immunoblot (Millipore). The PVDF membrane was activated in 100% methanol for
two minutes. Transfer occurred overnight at 180 mAmps, +4°C, in Tris-Glycine transfer buffer (Bio-Rad). After transfer, the PVDF membrane was placed in a fixing buffer (99ml of TBS with 1ml of 50% Glyceraldehyde (Sigma)), and rocked for 30 minutes. For immuno-detection the membranes were processed with a Western Breeze Kit (Invitrogen). ImageJ and Sigma gel image processing software were used to quantify relative protein densitometry.

From identical sister gels, bands of interest located at the 10-15 kDA region were then isolated for In-gel tryptic digestion and protein identification by mass spectrometry. In this setting, αHD5 was recovered from effluent samples and subjected to PAGE as described above. Gels were sliced and then washed with 100 mM ammonium bicarbonate and incubated with 50 mM ammonium bicarbonate, 1 mM DTT at 60°C for 30 min. The tubes were cooled to room temp and iodoacetamide was added to 1 mM and incubated further for 30 min in the dark, at room temperature. The solvent was discarded and the gel slices were washed in 50% acetonitrile/100 mM ammonium bicarbonate. Subsequently, the gel slices were transferred onto a 96 well Montage plate (Millipore) and destained with 50% acetonitrile in 25 mM ammonium bicarbonate, dehydrated with acetonitrile for 5 minutes, and vacuum dried. Gel pieces were then rehydrated in 25 mM ammonium bicarbonate, 10% acetonitrile supplemented with trypsin (5 μg/ml, Promega, Madison, WI, USA) at 37°C for 16 hours. Afterwards, tryptic peptides were extracted in 0.1% Trifluoro acetic acid/50% acetonitrile and mixed with equal volume of 5mg/ml α-Cyano-4-hydroxycinnamic acid (Acros Organics, New Jersey, USA). Tandem mass spectra were recorded with a matrix assisted laser desorption/ionization–time of flight, time of flight (MALDI-TOF-TOF) spectrometer (4800 Proteomics Analyzer, Framingham, MA, USA) set in reflector positive mode. The samples were ionized with a fixed LASER intensity of 3800J and
1000 LASER shots were collected per sub-spectrum and were shot randomly with uniform bias. The detector voltage was 2.1KV, the bin size was set at 0.5ns and the Signal/Noise threshold was set at 15. The spectra were collected with a specified mass range of 700-4000 Daltons with a focus mass of 2100 Daltons. MSMS ion search was performed through the SWISS-PROT database using MASCOT (Matrix Science). The search parameters were set as follows: cysteines as carbamidomethyl derivative, allowed peptide mass error 50 ppm, at least four peptides mass hits required for protein match, up to one missed cleavage and methionine oxidized form.

It was from this molecular MALDI-TOF-TOF analysis that we were able to confirm the presence of the pre-pro peptide form or zymogen of αHD5 in the NOD2 mutant effluent. These results suggest that the Paneth cell, despite donor genetic origin secondary to stem cell carry over in the initial engraftment, no longer maintains a wild type profile. Here, the reduction in αHD5 quantity and the altered maturity or quality of the αHD5 peptide is no longer representative of a NOD2 wild type LPDC containing graft. This indeed may advocate for a hierarchical role of the LPDC in dictating the maturity of the Paneth cell and its products in that a NOD2 wild type LPDC/ NOD2 wild type graft express mature 3.5kDa αHD5 and do not present with adherent microbes. Alternatively, a NOD2 mutant LPDC/NOD2 wild type graft express primarily a ~10 kDa pre-pro-form of the peptide which does not display the same cationic residues of the functional dimeric binding site needed for pore formation.

Considering the importance of Wnt in the expression status of the Paneth cell’s anti-microbial peptide αHD5, we asked whether the Paneth cells from allografts carrying NOD2 mutant LPDCs exhibited evidence of Wnt “under-stimulation” and subsequent inability to provide proper anti-microbial peptide production and protection of the mucosal epithelium. We
first assessed these measures *in vivo*, by determining the extent to which the Paneth cell had been Wnt stimulated by examining the phosphorylation state of Disheveled-2 (Dvl-2) a phosphoprotein which acts downstream of the activated Frizzled receptor in both the canonical and non-canonical Wnt pathways (Figure 11 B). This activation process involves the binding of Wnt to Frizzled (Fzd) leading to the poly-phosphorylation of Dvl-2 → Dvl-2(P). This poly-phosphorylated Dvl-2(P) then migrates from the plasma membrane into the cytoplasm where it forms “punctate bodies,” which can be clearly seen microscopically (29). When we examined allograft tissue specimens from epithelium in contact with the NOD2 wild type CX3CR1\(^+\) LPDC, Dvl-2 could be clearly recognized in its Dvl-2(P) activated-punctate state (Figure 11 B a), suggesting that the Paneth cells from these tissues were receiving Wnt stimulation *in vivo*.

In contrast, epithelium in communication with the NOD2 mutant CX3CR1\(^+\) LPDC displayed weak expression of “activated” Dvl-2 (Figure 11 B b,c) by immuno-fluorescence, and were confirmed at the protein level by immunoblotting of the poly-phosphorylated Dvl-2(P) protein (Figure 11 B e). Understanding that these findings could be due to either under expression of the gene, leading to less Dvl-2 protein production or alternatively, the lack of Wnt activation leading to depressed poly-phosphorylation of the protein intermediate, we chose to evaluate the mRNA quantity expression status of the individual epithelium isolates in order to determine the cause of lower Dvl-2(P) punctuation.

After evaluating relative Dvl-2 mRNA quantities, the abundance of Dvl-2 mRNA was found to be similar in epithelium in communication with either NOD2 wild type or mutant CX3CR1\(^+\) LPDC (Figure 11 B d). This suggests that there is not an intrinsic defect within the epithelium itself leading to reduced gene expression of Dvl-2 but rather there being a void of
proper Wnt stimulation to the epithelium in the graft in contact with the NOD2 mutant LPDC.

The discovery that the expression of Wnt5a was significantly reduced in the NOD2 mutant CX3CR1+ LPDCs led us to examine how this particular Wnt ligand, Wnt5a, could influence the transcription of αHD5. Wnt5a is considered a “non-canonical” member of the Wnt family, in that it triggers a transcriptional program by modulating intracellular calcium rather than by altering the intracellular disposition of beta-catenin, which defines the outcome of the classical canonical pathway. However, recent research has questioned whether Wnt5a may indeed play a molecular role in both non-canonical and canonical signaling pathways. However, at this time canonical Wnt ligands are believed to activate the αHD5 gene expression through the transcription factor TCF4, which is carried into the nucleus among a complex with beta-catenin (25).

Knowing the reliance the Paneth cell has on Wnt, we challenged isolated crypts with recombinant Wnt5a and monitored expression levels of TCF4 and αHD5 mRNA using RT-PCR (Figure 11 C). From these studies, we determined that the abundance of TCF4 mRNA isolated from untreated crypts recovered from allografts populated by NOD2 mutant LPDCs was significantly depressed (Figure 11 C, NOD2 MT, T=0) compared with crypts recovered from allografts with NOD2 wild type LPDCs (Figure 11 C, NOD2 WT, T=0). Addition of exogenous Wnt5a stimulated expression of TCF4 in isolated intestinal epithelium from both groups, consistent with the hypothesis that the donor allograft epithelium has the potential to respond to Wnt5a, independent of the NOD2 genotype of the LPDC (Figure 11 C). In contrast, as anticipated, Wnt5a addition alone was not sufficient to induce expression of epithelial αHD5
mRNA under the conditions of the study, suggesting that Wnt5a is not a direct first order substrate for αHD5 production.

These data would suggest that TCF4 transcription can be regulated through a non-canonical Wnt pathway and that the lack of Wnt5a expression by the LPDC could in fact be a cause of the depressed TCF4 levels within the Paneth cell.
Figure 11. Epithelial activity as it relates to the NOD2 genotype of the CX3CR1+ LPDC.
A. αHD5 expression in Paneth cells. Representative allograft ileal biopsies from a NOD2 wild type (panel Aa) and a heterozygotic NOD2 mutant recipient (panel Ab) visualized by immunofluorescence to detect Paneth cell αHD5 (αHD5 (Cy3, red)); DNA (DAPI, blue). Ac. Averaged αHD5 fluorescence of images from 11 NOD2 wild type and 19 NOD2 mutant patient samples. Ad. Relative αHD5 mRNA abundance in biopsy samples, as analyzed by RTPCR. Ae. (inset) Representative western blot demonstrating mature αHD5 in protein extracted from biopsies and average relative protein densitometry from αHD5 peptide immunoblots from 30 patients. B. Paneth cell Dvl-2-activation assessed in an ileal biopsy from (Ba) NOD2 wild type or (Bb) heterozygotic NOD2 mutant recipient. The lower edge of the crypt is marked with arrows. Dvl-2 activation is detected by the appearance of immuno-fluorescent punctate bodies detected by an antibody specific for phosphorylated Dvl-2. Bc. Averaged FITC fluorescence of images from 11 NOD2 wild type and 19 NOD2 mutant patient samples. Bd. Relative Dvl-2 mRNA abundance in biopsy samples, as analyzed by RT-PCR. Be. Relative densitometric analysis of Dvl-2 immunoblots averaged from all patient samples; (inset) Representative immunoblot of phosphorylated Dvl-2, extracted from two ileal biopsies. C. Stimulation of epithelial TCF4 by Wnt5a. mRNA abundance determined by RT-PCR. D. Proposed circuit involving microbial adherence and CX3CR1+ LPDC Wnt5a secretion. (Scale bar, 10 microns). NS, not significant. (*) indicates P value <0.05.

Table 2. Mass Spectroscopic analysis of the two 10 kDa αHD5 precursor forms found in grafts luminal effluent of the NOD2 mutant LPDC containing while not present in NOD2 wild type LPDC containing grafts. In gel tryptic digestion was performed on immuno-blots to obtain electrophoresed peptides following ethanol precipitation of the effluent supernatant.

<table>
<thead>
<tr>
<th>Protein Name Accession Number</th>
<th>Protein MW</th>
<th>Protein PI</th>
<th>Total Ion Score</th>
<th>Total Ion Score C.I.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q01523</td>
<td>DEF5_HUMAN Defensin-5 precursor - Homo sapiens</td>
<td>10065.00977</td>
<td>8.3</td>
<td>83.73</td>
</tr>
<tr>
<td>Q01523</td>
<td>DEF5_HUMAN Defensin-5 precursor - Homo sapiens</td>
<td>10065.00977</td>
<td>8.3</td>
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</table>
To extend these observations to the living organ, we examined the 2 transplants performed at our institution over the last 6 years that were retrospectively found to have derived from NOD2 mutant donors and were transplanted into wild type recipients. αHD5 mRNA abundance obtained from the organ at time of donation was compared to that measured several months after surgery, by which time the organ had been repopulated with NOD2 wild type dendritic cells of the recipient. In each of the 2 patients, the abundance of αHD5 mRNA had almost doubled, comparable to values measured in organs from NOD2 wild type donors that had been implanted into NOD2 wild type recipients (Table 3).

Table 3. Recovery of Paneth cell function in a NOD2 mutant bowel following transplantation into a NOD2 wild type recipient. QRTPCR Taq-man assay of total αHD5 mRNA extracted from isolated crypts and conducted as described in Methods. Tissue specimens were recovered from allograft biopsies before engraftment and several months following transplantation, demonstrating recovery of αHD5 mRNA abundance in a NOD2 mutant allograft following engraftment into a NOD2 wild type recipient. mRNA was quantitated as previously described. Significance was calculated by a t-test. (WT, wild type).

<table>
<thead>
<tr>
<th>CX3CR1 LPDC NOD2 Status</th>
<th>Epithelial NOD2 Status</th>
<th>HD5 copies/ng pre-engraftment</th>
<th>HD5 copies/ng chimeric tissue</th>
<th>Months Out</th>
<th>Fold Change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>3323</td>
<td>3474</td>
<td>20</td>
<td>1.045</td>
<td>0.82</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>2981</td>
<td>2905</td>
<td>27</td>
<td>0.975</td>
<td>0.614</td>
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<tr>
<td>WT</td>
<td>WT</td>
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<td>3594</td>
<td>10</td>
<td>1.022</td>
<td>0.736</td>
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<td>3481</td>
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<td>2.215</td>
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<tr>
<td>WT</td>
<td>1007 -/-+</td>
<td>1471</td>
<td>3714</td>
<td>37</td>
<td>2.523</td>
<td>0.006</td>
</tr>
</tbody>
</table>

To explain the studies that we have reported here, we propose the following model (Figure 10 D). The CX3CR1+ LPDC is depicted as a “sentinel” cell, responding to the presence of microbes at the epithelial surface of the crypt-villus axis. Here the CX3CR1+ LPDC extends
distinctive trans-epithelial dendrites from the body of the leukocyte through tight junctions of the adjoining enterocytes in order to safely access the lumen of the small bowel without there being increased permeability of the barrier. This TED extension is directly related to the expression of the CX3CR1 receptor and its ability to bind the enterocyte produced CX3CL1 chemokine-ligand. Here, these cells act as truly efficient antigen presenting cells who are capable of sensing bacteria within the lumen or on the mucus layer which is adherent to the epithelial mucosa. Within this mucus layer, high concentrations of the mature form of αHD5 exist, which when in contact with the cell wall of a bacterium, permit the formation of a multi-meric pore and thus destroy the organism through alterations in its membrane potential. Bacteria and their components are then taken up by the TED of the LPDC and processed via a wide array of Toll-like receptors, NOD and NOD-like receptors. Here the mutational SNPs in NOD2 are thought to produce their deleterious effect in that they ineffectively bind MDP and cannot perpetuate a signal response to the NF-κB pathway. With proper activation of NOD2, within a wild type DC, multiple pathways are thought to correctly orchestrate certain cytokines leading to an extracellular response toward organisms in addition to expressing the inflammatory Wnt5a ligand. Here, expression of Wnt5a, is thought to stimulate the expression of αHD5 by increasing the levels of the key αHD5 transcription factor, TCF4. This increase in TCF4 expression is thought to prime the Paneth cell’s ability to then produce more αHD5 upon TCF4 activation and translocation. Degranulation of the immature form of the αHD5 peptide leads to trypsin processing of the αHD5 peptide in order to present the mature active form of αHD5, which we see in NOD2 wild type patients.
Within the defective circuit we describe here, a mutation in the intra-cytoplasmic receptor NOD2, induces an inability of a distinct APC to taste or sense microbial loads efficiently. Whether this inability to sense bacterial MDP results in pressure on other pathways or subsequent downstream defects within the NF-κB pathway, the LPDC is (1) left unable to extend its dendrites, (2) under expressing TLR, ICAM and MIF subfamily proteins, (3) improperly augmenting its expressive cytokine profile (4) unable to express normal values of Wnt5a when compared to wild type NOD2 controls. These finding put the NOD2 mutant chimeric bowel’s phenotype focus more on the inability of the LPDC to direct the epithelium rather than there being a true intrinsic defect in the stem cell derived epithelium of the NOD2 wild type donor, which appears to maintain the proper molecular machinery to activate the TCF4/αHD5 axis under in vitro Wnt5a stimulation. These findings, when extrapolated suggest that defects in the Crohn’s Disease patient’s bowel correlate more with a mutation in the bone marrow derived antigen presenting cell, namely the sentinel LPDC, rather than in the epithelium itself.

**DISCUSSION**

The data we have reported here demonstrate that the human intestinal CX3CR1+ LPDC bearing a Crohn’s disease associated NOD2 genotype displays a profoundly different phenotype than its NOD2 wild type counterpart both within the allograft and when challenged in vitro. Our study utilizes LPDCs isolated directly from human intestine. This model permits examination of
the phenotype of the recipient derived differentiated cell that has taken up residence in the lamina propria of the donor derived graft. Our prior work suggests that a NOD2 expressing cell of bone marrow origin must be involved, since the epithelium remains donor in origin secondary to the Lgr5+ stem cell within the crypt. From our recent findings we suggest the NOD2 defective CX3CR1+ LPDC is not only phenotypically defective, but that its inability to express proper levels of Wnt5a could explain the deficiency seen in the Paneth Cell production of αHD5 secondary to TCF4 under expression. Further studies and more sophisticated models are needed to prove this connection; however, the transplanted human intestine certainly provides an interface where NOD2 defective dendritic cells can be shown to alter the state of the mucosal innate immunity niche. These data depict the (1) failure of the LPDC cell to extend dendritic processes and suggests that one of this cell’s key roles, namely the monitoring of the micro-environment on the lumenal surface is impaired (2) The CX3CR1+ LPDC produces an altered expression pattern with regards to TLR, ICAM and MIF subfamily proteins as well as overproduce IFNγ and TNFα resulting in improper adhesion, pattern recognition and cytokine production (3) Permit mucosal accumulation of luminal microbes at the intestinal barrier, resulting in penetration of the cellular compartment and (4) Secrete 14-fold lower levels of the Wnt5a peptide, a Paneth Cell maturation ligand.

These findings suggest that when a NOD2 wild type epithelium, which possess a NOD2 wild type Lgr5+ stem cell, is in contact with a recipient bone marrow derived NOD2 mutant CX3CR1+ LPDC, the once “normal” epithelium reverts back to an apparent Crohn’s profile. This is thought to occur secondary to the NOD2 defect on the natural function of the LPDC. This natural function depicts the CX3CR1+ LPDC as a cell that is able to utilize the information it
gathers through sampling of the micro-environment to help orchestrate the immune cells within the lamina propria to generate an appropriate response to the microbes that have approached or breached the epithelial barrier (2). Through SNP alterations in the allele and consequential structure modification and protein truncation, the NOD2 MDP receptor leaves the LPDC functionally blinded to potential microbial invasion of the epithelium by preventing the NOD2 receptors migration to the intracytoplasmic membrane to activate the NF-κB pathway. Without this sentinel cell directing proper innate immune response systems toward bacterial organisms, the tissue is left responding on its own accord and unprotected.

As yet we do not understand how the NOD2 defect causes the phenotypic lesion. We do know that both the CX3CR1 receptor and its ligand are present, so more distal components of the CX3CR1/CX3CL1 circuit must be involved. The CX3CR1+ LPDC originates from a bone marrow monocyte and requires GM-CSF stimulation as it journeys to the small intestine (31). Our data suggest, that in humans, NOD2 does not play a role in that portion of the maturation of this dendritic cell.

We have noted that the expression patterns of proteins important in dendritic cell function differ significantly between the NOD2 mutant and NOD2 wild type dendritic cells. The failure to express TLR2 should impair the response of the cell to microbes, in addition to the NOD2 defect (32). The increased expression of IFN-γ and TNF-α by the NOD2 mutant cell speaks to the possibility of heightened local inflammation, as if in the absence of a functional NOD2 locus the LPDC switches an altered baseline inflammatory circuit to protect the intestinal wall from microbial invasion, similar to what is seen in CD. We attribute the absence of pathological evidence of chronic inflammation in the allograft under these settings to the immunosuppressive
therapy administered. It is because of this suppression, that we can see the process drawn out until inflammatory forces are so great they overcome the immune suppression and the graft develops acute cellular rejection.

We were surprised to discover that a defective CX3CR1+ LPDC could be recovered from each of the recipients bearing a CD associated NOD2 polymorphism, regardless of the genotype. We had anticipated that a defect in LPDC function would likely be associated with those genotypes most at risk for CD, such as L1007fs, or homozygotes of any of the loci. It is clear that mutations in NOD2 represent risk factors that require other, yet unknown, factors to “tip the balance” in such a way as to cause inflammatory bowel disease. For example, a viral infection was recently reported to induce an inflammatory bowel phenotype in mice engineered with an ATG16L1 deficient genotype (33). It is possible that our transplant population has been “stressed” in some fashion (e.g., immunosuppression and/or subclinical viral infection) that exposes the dysfunctionality of the NOD2 mutant phenotype. Alternatively, the “background” genotypes of the patients in our sample might include genetic risk factors for intestinal failure that interact with the NOD2 lesion to impair the LPDC, since in each case the small bowel had not been functioning as a viable organ and had to be surgically removed.

We have reported here that Wnt5a is produced by the CX3CR1+ LPDC of the small intestine and both its expression and secretion appear to be dependent on NOD2 circuitry. Recent studies have suggested that Wnt5a plays a role in bridging innate and adaptive immunity (34). For example, macrophages stimulated with IFN-γ and endotoxin have been shown to express Wnt5a via a TLR dependent circuit. Wnt5a, in turn, stimulated expression by the activated macrophage of the pro-inflammatory genes IL6, IL1α, IL8, and MIP-1α (34). Should
the human CX3CR1+ LPDC utilize Wnt5a to modulate activity of immune cells within the intestinal lamina propria, this function might be expected to be impaired in the setting of a Crohn’s disease associated NOD2 mutation.

The role of Wnt5a in human gastrointestinal biology has not been well studied. Wnt5a has been reported to be present in the lamina propria of the human small intestine (30), although its cellular origin was not reported. The protein appears to play a key role in the development of the small intestine in that Wnt5a knockout mice exhibit a profound shortening of the midgut with duplicated segments, as well as defects in epithelial proliferation (35). Our finding that Wnt5a can stimulate the expression of TCF4 in the intestinal crypt epithelium, suggests that Wnt5a could normally influence the well-studied canonical Wnt pathway involved in intestinal epithelial growth and renewal by modulating levels of the a key transcription factor mobilized by Wnt ligand activation, altering the “gain” on the circuit.

Recent studies have implicated TCF4, a known regulator of Paneth cell differentiation and defensin expression, in the pathophysiology of Crohn’s disease (25, 26). The abundance of TCF4 mRNA in biopsy specimens collected from individuals with ileal Crohn’s disease were significantly decreased when compared to tissue specimens from colonic Crohn’s or ulcerative colitis. TCF4 mRNA levels positively correlated with mRNA levels of αHD5 and αHD6 in these samples (25). These data provide support for the hypothesis that inadequate Wnt-stimulated expression of Paneth cell defensins contributes to the pathophysiology of ileal Crohn’s disease. We suggest that Wnt5a secreted by the CX3CR1+ LPDC might contribute to Wnt-based stimulation of the Paneth cell through modulation of levels of TCF4.
We propose, based on data reported here, that the CX3CR1+ LPDC is a key intestinal cell whose normal function is dependent on NOD2, and is possibly compromised in certain forms of Crohn’s disease. The failure of the NOD2 lesioned LPDC to express Wnt5a provides incentive to more deeply examine the biological processes within the small intestine that might be influenced by Wnt5a deregulation in the setting of Crohn’s disease. Our data provide additional support for the hypotheses that propose that NOD2 mutations in Crohn’s disease diminish the strength of epithelial antimicrobial defense, but argue a key role for the CX3CR1+ LPDC in the mechanism.

**DIRECTION AND GOING FORWARD**

The investigation and analysis presented here spans decades of clinical and basic science research conducted on the disease first described by Burrill Bernard Crohn in 1932 as regional or “terminal ileitis” (1). Although there is still no commonly agreed upon cause among those who intently study the disease it is clear that the leading precipitating factors share a common theme in describing the “destruction of a mucosal barrier.” This destruction, whether it be caused by: (1) a pioneer microorganism, such as a mycobacterium avium sub-subspecies, who invade the mucosa and subsequently permit commensal manipulation of the mucosal niche or (2) a defective Paneth cell, incapable of translating proper anti-microbial peptides permitting microbial penetration or (3) there being a defective bone marrow derived antigen presenting
sentinel cell, incapable of sensing bacterial load due a defective NOD2 receptor, one thing is clear:

A once previously finely tuned circuit which once acted to protect the barrier has turned on itself.

Flooded with activated cytotoxic T-cell lymphocytes and cytokines, such as IFNγ and TNFα from the lamina propria compartment and luminal microbes from the epithelial compartment the intestinal wall is left helpless and in a state cellular chaos.

It is here, within this comparative chaos, the human intestinal transplant model can be of great value in that it has the capacity to separate the two compartments: epithelial and lamina propria in such a way that we can evaluate each on their own “protective merit.” As discussed earlier within the review of current literature and the productive primary research, the human intestinal allograft provides an epithelial platform, which will remain of donor genetic origin, while the lamina propria compartment, over time, will become re-populated with recipient bone marrow derived immune cells. This interface between donor epithelium and recipient immune cells allows one to focus on the communication or miscommunication that occurs between these cell populations when mutations in the NOD2 gene are introduced to the dichotomous cellular system (4) (5).

As depicted in the first figure of the primary integrated research, it becomes apparent that after 90 days of the NOD2 wild type donor intestinal allograft residing within the NOD2 mutant recipient, we see a discrete alteration in the physical phenotype of the CX3CR1+ LPDC. These dendritic cells, known for their ability to extend dendrites from the lamina propria across tight
junctions and into the lumen of the intestine, appear to no longer possess the capacity to facilitate this dynamic process. When comparing the wild type and mutant chimeras, both still appear to possess the translational capacity to express the CX3CR1 protein. However, this protein does not correlate with extended trans-epithelial dendrites, which can be assumed from the lack of F-actin organization within the mutant dendritic cell and the globular and/or pleomorphic phenotype. Overlap within the NOD2 wild type exists between the CX3CR1 (green/FITC) protein and F-actin (red/Cy3), which can be clearly seen in both the mono-filtered (Figure 3.b-c) and merged images, which depict the dendrite as yellow. From these representative images, we can assume that, although the mechanism remains unclear, there is disruption of the intracellular scaffolding within the NOD2 mutant dendritic cell. Whether this disruption is occurring due to intrinsic alterations of the actin cytoskeleton subsequent to NOD2/ NF-κB axis binding abnormalities or there being additional adhesion receptor abnormalities secondary to the NOD2 mutation, the data is still unclear. (6)

Understanding the lack of clarity or cause for this phenotypic abnormality within the mutant NOD2 dendritic cell, we are left only to speculate and suggest further research in the direct development of site directed mutagenesis within the DC itself, targeting the NOD2 alleles. One could suggest that mutations in the NOD2 gene remain “silent” in many cases unless one of the common IBD SNPs located at 702, 908 or 1007 results in the alteration of the NOD2/ NF-κB binding axis to actin, preventing F-actin polymerization. If this indeed were the case, one would argue the difficulty in the ability of the DC to extravasate from the blood, through the endothelial cell to the lamina propria and finally the sub-mucosal region. It is not merely the lack of TED extension within the lamina propria which questions the NOD2/ NF-κB binding axis hypothesis,
but rather how the DC itself migrated to this region without the capacity of extending F-actin mobilized dendrites, particularly when the NOD2 mutant DC itself still possess the ability to express the CX3CR1 fractalkine receptor.

These arguments suggest that there is something more complex residing within the defective CX3CR1+ LPDC unrelated to F-actin organization and CX3CR1 expression. In fact, the NOD2 mutant CX3CR1+ LPDC populations collected from our mutant recipient patients, when averaged, actually expressed more CX3CR1 at both the mRNA and protein levels. This finding would advocate for a hypothesis which would define the DC as having the proper F-actin cytoskeleton capable of migrating from the blood to the lamina propria in addition to having the correct CX3CR1 receptor to interact with the epithelial CX3CL1 ligand. The lack of TED extension would be a consequence of abnormalities within the DC transcriptome, particularly when evaluating ICAM and integrin expression, which we see within the NOD2 mutant DC (Figure 5 C). Here, the averaged values from a custom RT-PCR dendritic cell expression array showed reduced levels of ICAM1, ICAM2, integrin beta 2 and integrin alpha M among the NOD2 mutant dendritic cells when compared to NOD2 wild type dendritic cells. Understanding that both cell populations possess the ability to migrate from the blood to the lamina propria, suggesting proper F-actin migratory dynamics; and that both cells can equally express the CX3CR1 receptor, these findings of reduced ICAM and integrin expression suggest a NOD2 associated defect in the ability of the DC to initially micro-orient itself below the epithelium so as to properly align the CX3CR1/CX3CL1 fractalkine axis for TED extension.

Beyond, alterations in the physical phenotype of the dendritic cell when in possession of a mutant NOD2 allele, the cell also appears to express depressed levels of other pattern
recognition receptors, such as TLR 1 and TLR 2. These SNP defects in NOD2 have never been associated with TLR depression, although there is no current literature evaluating the NOD2 defect in the LPDC, suggesting that there may indeed be an inter-connection in pattern recognition receptor families in antigen presenting cells. Although the ligand for TLR2 is lipotechoic acid and MDP is the ligand for NOD2, studies have shown similar activation pathways of the two receptor axis when stimulated with IL-23 (7). Moreover, the end activation pathway is shared among the two pattern recognition receptor families in that both result in the activation of the NF-κB pathway. Could it be that the NOD2 mutation results in abnormal binding of the NF-κB complex, preventing proper TLR interaction with the keystone NF-κB intermediate? Some studies on periodontal microbial activation patterns on eukaryotic cells lines have shown that “TLR2 and NOD2 are functionally active receptors during innate immune responses to invading bacteria, and thus result in a combination of signaling through TLR and NOD leading to the synergistic enhancement of inflammatory reactions” (8) Could it be that this synergy is de-synergized when there is a fatal defect in NOD2?

Further studies on the defect in NOD2 among other pattern recognition receptors is drastically needed in order to determine the true interdependence of the two families on one another. Although SNPs in TLR family members have not been associated with the development of Crohn’s Disease at this time, it would certainly be beneficial to the field to define the method of interaction between the two bacterial receptors. Perhaps a NOD2 defect mutation could be overcome by hyper-stimulating the TLR subfamily members in hope to overcome a possible NF-κB blockade by NOD2 defective binding. These studies could be initiated within a NOD2 mutant
murine model which does possess the NOD series receptors and members of the Toll-like receptors.

By defining the LPDC defect with regard to its apparent alteration on both the phenotype of the cell and the expression profile, we are able to make sense of the common finding surrounding bacterial mucosal adhesion within Crohn’s Disease. With an LPDC unable to extend its TED and inability to activate NOD2, one would assume that bacteria would accumulate at the mucosal wall of the intestine and that the epithelium itself would be stressed by the toxic bi-products of the micro-organisms and their potential virulent factors. Indeed we see markedly increased bacterial adhesion and even infiltration into the mucosa with the 16S rRNA bacterial probe EUB338, although we are left without knowing the type of organisms that are predominately adherent in this situation. We could consider our previous research on the small intestinal microbiome in transplant patients which indicates an inversion of what is typically found. Our studies here have shown that the transplanted bowel, when possessing an ileostomy, harbors greater numbers of enterobacteriaceae and lactobacilli rather than bacteroides and clostridia, who are characteristically the organisms of the terminal ileum (Figure 1). However, these organisms are not particularly virulent or invasive in nature. Understanding this, one might assume there is some sort of pioneer organism that initially penetrates the mucosal barrier in order to upset the natural order of the innate mucosal immune system and permit other commensals to attach.
Figure. 1. Summary of the relative bacterial populations found under various clinical conditions. In each chart, the population of each of the four bacterial orders is shown as the geometric mean of its percentage of the total population. (A) Ileal effluent from transplant patients, ileostomy present. (B) Ileal effluent from non-transplant patients, ileostomy present. (C) Biopsy samples from transplant patients, ileostomy present. (D) Ileal effluent from transplant patients during transplant rejection, ileostomy present. (E) Ileal effluent from transplant patients, ileostomy closed. (F) Regraphed selected from biopsies of the terminal ileum of six healthy subjects. (Figure taken in its entirety from Reference 12)
Initial 16S rRNA sequencing data from studies in our lab and in conjunction with the University of New South Whales has found that when comparing the effluent of NOD2 mutant patients to NOD2 wild type, those who possessed the mutation often carried species of Campylobacter and Helicobacter that were not seen in wild type patients. Whether these organisms are “niche” organisms that have pioneered invasion of the mucosa is still to be determined, however knowing the mechanism of H.pylori’s ability to disrupt protective mucus layers in the stomach could suggest a preemptive role in initiating gut inflammation in patients with the NOD2 defect.

![Figure 2. A Potential Pioneer Organism.](image)

**Figure 2. A Potential Pioneer Organism.** Summary of potential invasive pioneer bacterial organisms sequenced from 11 NOD2 mutant transplant patients effluent at the time of ileostomy closure, approximately 120 days post-transplant. The organisms total RNA was isolated from the stool and sequenced using multiple universal 16S rRNA primers and evaluating hyper-variable regions of the sequence to determine speciation. Campylobacter (C), Helicobacter (H) base pair (bp).

Although there is much research to be done on the topic, the idea that there may be a definitive type of organism or bacterial species initiating the adhesion or invasion we see within the NOD2 mutant, it is clear that the epithelium is stressed by their presence. As depicted in the results, mRNA and protein expression of the fractalkine stress ligand is 5 and 10 fold higher in the mutant respectively than it is in the wild type. These findings suggest that the cellular entities
of the epithelium, namely the enterocytes, are calling out for help to those cells that possess the CX3CR1 receptor.

With the knowledge that the NOD2 mutant dendritic cells do indeed express more CX3CR1 than the wild type, we could assume that there is either (1) a structural defect in the CX3CR1/CX3CL1 axis or (2) that the NOD2 mutation creates an intracellular blockade preventing CX3CR1 from initiating an interaction with the CX3CL1 ligand. This could be studied by binding kinetics assays using the Biacore ® surface plasmon resonance chip system. Here, the CX3CL1 ligand peptide could be placed on the gold bed of the chip and either free CX3CR1 from the LPDC or the CX3CR1+ LPDC itself could be streamed over the ligand bound chip in order to determine binding kinetics of both NOD2 wild type and NOD2 mutant derived systems. From these studies, one could determine if a NOD2 SNP directly alters the binding efficacy of the CX3CR1/CX3CL1 axis itself or whether there is indeed another variable preventing the cell from extending dendrites despite increased CX3 receptor and ligand.

Considering that CX3CR1+ LPDC NOD2 mutant receptor is unable to bind MDP properly and is in turn defective in properly activating the NF-κB pathway we begin to question the role of Wnt5a in this defective dendritic cell inflammatory activation circuit. Understanding that “Wnt5a can control cell orientation, polarity, and directional movement in response to positional cues from chemokine gradients” we begin to question if the dendritic cell’s abnormal phenotype, in which it cannot extend TEDs, may be in fact be compounded secondary to the NOD2 defect.

Some have suggested that Wnt5a is up regulated in environments with increased levels of IL-1, IFNγ and TNFα, suggesting that the Wnt ligand is not only an inflammatory ligand itself
but also a reactive inflammatory species to other inflammatory molecules (10). These studies would suggest that Wnt5a should be up regulated in our NOD2 mutant dendritic cell chimeric tissues since there are elevated levels of these cytokines. This observation may in fact be true in a cell which has the ability to react to microbial products, as published in 2006 by Blumenthal. However, as shown in this study and in the Blumenthal publication, the antigen presenting cell’s expression of Wnt5a as a reactive product to inflammation is dependent on an intact NF-κB signaling pathway, which a SNP in NOD2 does in fact alter and prevent from progressing. Our findings, which suggest a defect in NOD2 lead to altered Wnt5a expression by the dendritic cell, correlate exactly with the profile Blumenthal depicts in his publication: “The Wingless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation.”

Follow up in-depth studies of this suggested mechanism are needed in order to truly elucidate the exact mechanisms of Wnt5a depression in the NOD2 mutant leukocyte. Initial studies could involve introducing a specific SNP into the NOD2 allele in order to define the NOD2 mutation and standardize the cell entity compared to its control wild type. Focused or genome wide array analysis could assist in developing an understanding of the deviation from baseline in the Wnt pathway when compared to an unaltered control.

In order to determine how the dendritic cell, with a NOD2 SNP, modifies the interface of the small intestine and the ability of the Paneth Cell to prime or potentiate the production of more Human Defensin 5, a murine system could be developed where a mouse CompoZr Knockout Zinc Finger Nucleases (ZFNs) Sigma ® targets the NOD2 gene. Here, the bone marrow from this mouse could be harvested and implanted into a genetically unaltered irradiated
clone, which upon repopulation of the intestinal lamina propria would result in a parallel chimeric model to our human intestine transplant patients. This murine model will however control for the epigenetic variation that exists between our patient cohort, in that these mice will be essential clones with NOD2 mutant CX3CR1+ LPDCs and wild type intestinal epithelium/Paneth Cells. Although mice do not express the exact αHD5 defensin that humans do, they do express the alpha defensin Cryptidin from the Paneth cell of their terminal ileum. Moreover, these Cryptidins have been shown to be reduced in mice with NOD2 mutations (12). While not an exact parallel model system, the mouse chimeric intestine would certainly control for the numerous variables concerning immune suppressive reagents and intestinal surgery that we see in the human transplant model.

While using the Ingenuity Systems ® visual pathway software and reviewing the potential interaction processes that exist between the NOD2 receptor, fractalkine axis and the Wnt5a stimulatory function, it appears to be likely that these pathways have multiple cytokine and NF-κB conduit systems that can certainly interact within inflammatory circuits such as those found in the bowel. Beginning with the NOD2 circuit of interactions, we can see there is more to the receptor than simple MDP binding and subsequent activation of NF-κB (Figure 3)
Figure 3. Diagrammatic depiction of (Left) the protein interaction that takes place when MDP, a component of peptidoglycan binds the intracytoplasmic receptor NOD2, leading to RIP2 interaction and NF-κB conversion to the transcriptionally active IκB. (Right) A circular diagram of interactions between NOD2 (center) and a spectrum of other receptors, pathways and cytokines. Diagram developed by Ingenuity Systems ®

Beyond the direct MDP/NOD2 activation pathway interaction described, there is also an abundant variety of other potential regulation pathways that the seemingly simple system may be involved with regard to regulation of the inflammatory response to microbes within the bowel. It is clear from this diagram that multiple forms of cytokines, pattern recognition receptors and kinase pathways can be either directly or indirectly affected by the function and expression of the
NOD2 receptor, suggesting that alterations in the inflammatory profile of the gut is highly intertwined with the ability to sense MDP by antigen presenting cells.

Focusing on the CX3CR1+ LPDC’s ability to express NOD2, we can see from the designated protein pathway and published interactions pathways that, here too, many cytokine driven systems are involved in the dendritic cell ability to function as a true antigen presenting

Figure 4. Diagrammatic depiction of the (Left) the intracellular pathway interaction that takes place when the CX3CR1 G-coupled receptor binds the enterocyte derived CX3CL1 ligand and successive NF-κB conversion to the transcriptionally active IκB. (Right) A circular diagram of interactions between CX3CR1 (center) and a spectrum of other receptors, pathways and cytokines. Diagram developed by Ingenuity Systems ®
sentinel within the gut. Furthermore, with a single defect, multiple intermediates can be changed and ultimately the NF-κB activation status can be manipulated (Figure 4).

![Diagram](image)

**Figure 5.** Depiction of a circular diagram of interactions between CX3CL1 (center) and a spectrum of other receptors, pathways and cytokines involved in regulation and augmentation of CX3CL1. Diagram developed by Ingenuity Systems ®

The ligand to CX3CR1, CX3CL1, has not only the ability to act as an adhesion peptide but also as a chemokine, which as depicted in (Figure 5), can activate everything from F-actin assembly and NF-κB, both important pathways in the dendritic cell, to other cytokines such as IFNγ and TNFα. Again, alterations in this system could be studied by first inducing a NOD2 knockout in a murine model for which CX3CL1 activation could be observed, followed by a
reversal CX3CL1 knockout to compare the gene effects on the tertiary signaling systems. Perhaps it takes manipulation in both NOD2 and CX3CR1/CX3CL1 systems to prevent proper F-actin coordination and TED extension within the LPDC.

Figure 6. Diagrammatic depiction of the (Left) the protein interaction that takes place when Wnt5a binds the FZD receptor initiating the non-canonical Wnt pathway which ultimately involves the regulation of NFAT, an important DC transcription regulator. (Right) A circular diagram of interactions between Wnt5a (center) and a spectrum of other receptors, pathways and cytokines. The interaction between Wnt5a and TCF4 is magnified. Diagram developed by Ingenuity Systems.

Wnt5a, a Wnt ligand recently discovered to act through both canonical and non-canonical mechanisms remains an intriguing player in inflammation in that its secretion has been associated with MDP activation of antigen presenting cells. Classically, Wnt was thought of as a
cell growth promoter and cell maturation inducer in which concentration gradients of the Wnt family could lead to the differentiation, polarization and migration of cellular progenitors in order to develop more complex organismal systems. Through attraction/repulsion gradient devised environments, axons and dendrites of the nervous systems and antigen presenting cells of the immune system are capable of altering their cellular morphology.

**Figure 7.** Depiction of (Left) a circular diagram of interactions between TCF4 (center) and a spectrum of other receptors, pathways and cytokines (Right) A circular diagram of interactions between Defensin alpha 5 or also known as DEFA5 or αHD5 (center) and a limited spectrum of other receptors, pathways, proteases and bacterial patterned components. The two diagrams both indicate the Beta Catenin Pathway (CTNNB1) as involved their mechanism (one o’clock position of both diagrams). Here TCF4 feeds into the CTNNB1 pathway while αHD5 or DEFA5 is effected by the CTNNB1 pathway confirming the TCF4→αHD5 transcriptional regulation in the literature. Diagram developed by Ingenuity Systems ®
However, with recent findings suggesting that Wnt5a can act as an inflammatory Wnt ligand, direct observations in a Wnt5a knockout murine model is needed. From these models, one could directly observe how the presence of micro-organisms alter the Wnt5a gradient within the mucosa of the bowel and its effects in the Paneth cells ability to mature and produce anti-microbial peptides in response to accumulating bacteria.

It is from this diagram of published Wnt5a interactions and our data, that we suggest that this Wnt ligand has not only the ability to specifically acts as an inflammatory signaling molecule but that it also plays a unique role in augmenting TCF4. TCF4, as stated, is the primary transcription factor responsible for αHD5 up-regulation. Increasing TCF4 levels subsequently led to the proper production of anti-microbial αHD5 peptide production within the terminal ileum’s mucosal Paneth cells through the use of the Beta Catenin pathway (CTNNB1) as depicted in Figure 7.

Finally, the small intestine is a unique organ in that it not only provides much of the nutrient extraction needed for a human being to live, it also possess the largest body of lymphatic tissue aggregate in a human. This accumulation of immune tissue allows for the intestine to “taste” or “sample” not only all of the antigens presented within what we consume as food but also permits the surveillance of the $10^{14}$ bacterial organisms that live within us on any given day. It is here within the terminal ileum that we can see what happens to the immune system when key regulatory components, such as NOD2, are altered in such a way that permits these organisms to overwhelm our body. Although NOD2, is not the only mechanism for sensing bacterial load within the human gut, when it is damaged, deregulated cytokine chaos ensues allowing a spectrum of potent inflammatory cytokines to alter the intestinal niche and direct the
body toward self-destructive inflammation, resulting in damage to the bowel wall. Beyond, the incessant inflammatory profile seen in Crohn’s Disease, the NOD2 defect also appears to alter the production of our own self-made anti-microbial peptides, which keep commensal organisms at a healthy distance from the mucosal epithelium. From this study and those that have preceded it, it is no surprise that there is no single pathological cause to Crohn’s Disease. We hope that the circuit shown by this human intestinal transplant study continues to stimulate the research of others in further elucidating all of the key molecular and cellular players within NOD2 effect on the dendritic cell, Paneth cell and Wnt5a pathway. Furthermore, we hope that one day a target for therapy can be developed and Crohn’s Disease can be cured.
APPENDIX A:
REFERENCES FOR LITERATURE REVIEW:


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APPENDIX B: REFERENCES TO PRIMARY RESEARCH AND FOCUSED DISCUSSION


APPENDIX C:
REFERENCES TO WHAT WE KNOW AND GOING FORWARD: A DISCUSSION OF RELEVANCE

APPENDIX D:
REFERENCES TO EXTENSIVE PROTOCOLS WITHIN MATERIALS AND METHODS:


AUTHOR CONTRIBUTION TO PRIMARY RESEARCH

Denver Lough MD PhD Candidate 2012, developed the research agenda for this project including the assembly of protocols, methods, experimental practice as well as manuscript and figure construction.

Joe Abdo MS, was a technician in the Small Intestinal Transplant Laboratory and during his time assisted in tissue tissue and dendritic cell acquisition.

Juan Guerra MD, was a Research Fellow in the Small Intestinal Transplant Laboratory and during his time assisted in tissue tissue and dendritic cell acquisition

Michael Zasloff MD PhD, provided tremendous mentorship, project development and guidance to the project as well as manuscript and figure development.

Thomas Fishbein MD, conducted all necessary human transplants and provided clinical insight to the project as well as assited in manuscript and figure development
This study was conducted under:

Title: Clinical Investigation of Intestinal Failure and Transplantation

IRB#: 2004-008