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Host-microbiota interactions: effects of *Nod2* deficiency

Dottorando: **Antonello Amendola**

Scientific Tutors:

Prof.ssa Rosa Sorrentino
Dipartimento di Biologia e Biotecnologie “C. Darwin”
Università di Roma “ La Sapienza”

Dott.ssa Monica Boirivant
Dipartimento di Malattie Infettive Parassitarie e Immunomediate
Istituto Superiore di Sanità – Roma

Board of examiners:

Prof. Carlo Cogoni
Dipartimento Biotecnologie cellulari ed Ematologia
Università di Roma “La Sapienza”

Prof.ssa Michela Ori
Dipartimento di Biologia
Università di Pisa

Dott.ssa Maria Cristina Gagliardi
Dipartimento Malattie Infettive Parassitarie e Immunomediate
Istituto Superiore di Sanità – Roma

Index

1. Introduction

The mucosa of the gastrointestinal tract is, in the human body, the widest surface of contact with the external environment. Under normal conditions its luminal content is represented by a mixture of combined nourishing elements and bacterial flora. A single layer of epithelial cells separates the external environment from that inside, characterized by the presence of cells belonging to the immune system strategically organized below the epithelial barrier to point out the presence of a continuous information interchange among the two environments. The epithelial barrier actively participates in the information interchange among external and internal environment. The organization of the local immune response is such to be able to discriminate among the signals coming from the presence of the normal microbial flora and those of danger represented by the presence of pathogens. From this it primarily follows the development of a tolerogenic immune-response in the case of signals coming from the normal microbial flora or the development of an sustained inflammatory response in the case of signals coming from pathogens to accomplish their elimination.

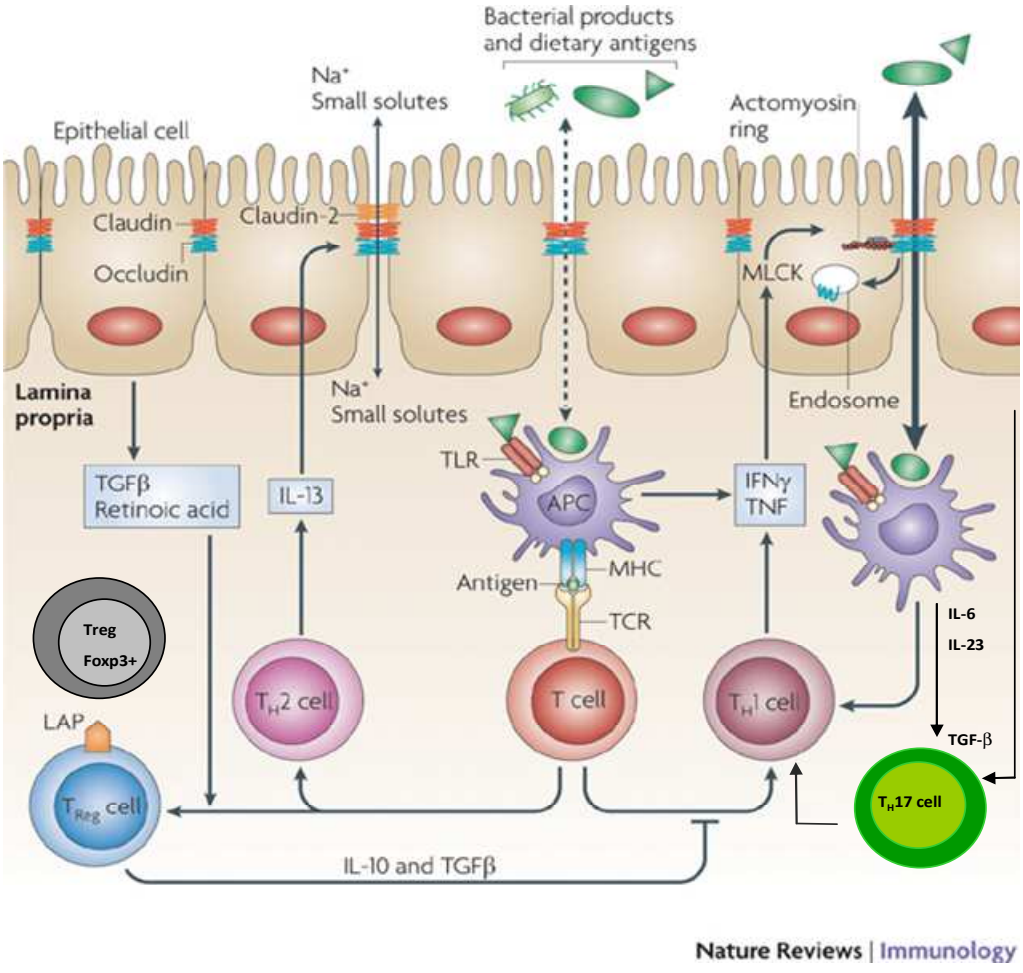


Figure 1. Intestinal mucosa components participating in the host-microbiota interactions. (Turner J.R. *Nature Reviews Immunology* 2009; 9, 799-809).

1.1 Mucosal Homeostasis

1.1.1 Intestinal Microbiota

The human intestine constitutes one of the microbial ecosystems with great density of population presents in nature. The human intestinal microbiota, with a quantity that ranges around 10^{13}-10^{14} microorganisms/ g colonic content with a biomass > 1 kg, outnumbers the microbial communities attached to the other surfaces of the human body and they are about 10 times higher than the total number of our somatic and germinal cells. (Savage D.C., 1977). Thus, the intestinal microbiota of humans accounts for a number of genes 100 times higher than human genome (Bäckhed F. *et al.*, 2005). The greatest part of these microorganisms are found in the colon where the densities of population specifically reaches values of 10^{11}-10^{12} microorganisms / ml of luminal content. Therefore, the human being can be considered a super organism made up by human and microbial cells, with a genetic unit constituted by the whole genes present in the human genome and in the genome of the intestinal microbiota (microbioma) (Lederberg J., 2000). The composition of the intestinal microflora depends on the number and on the type of microorganisms with which the subject comes into contact from the birth and in the first years of life and on the genetic background of the same subject. The variety of environmental interactions and the stochasticity of the microbial populations with which the subject comes into contact make the microbiota of a single subject exclusive. In the context of a specific intestinal habitat, some microbial components are autochthonous or "residents", while others, so called allochthonous components, originate from the ingested food, from the water or from other elements present in the external environment (pollutants) and this tight interactions makes this ecosystem very dynamic. The relationship that is established between the host and components of the microbiota has often been described as commensalism (a partner draws benefit while the other doesn't result influenced), but it is surely more appropriated to define it as symbiotic or mutual (both the partners increase their fitness).

The bacteria, fungi, protozoa, and viruses that inhabit the gastrointestinal tract combine to comprise the host's intestinal microbiota and are integral to digestive and metabolic processes necessary for general health. Our knowledge of the composition of the human gut microbiota was limited to culture-based studies until recent initiatives utilizing high-throughput sequencing and molecular phylogenetic approaches based on sequencing bacterial 16S rRNA genes made available a detailed inventory of the normal human gut microbiota (Eckburg P.B. *et al.*, 2005; Palmer C. *et al.*, 2007; Xu J. *et al.*, 2007; Zoetendal E.G. *et al.*, 2002). Such comprehensive enumeration studies of microbial diversity within the mammalian gut not only improved our understanding of the population composition and the dynamics and ecology of the gut micro-biota but also provided evolutionary insight into the host–microbe mutualism. Although there are at least 55 divisions of bacteria and 13 different divisions of archaea on Earth, the human distal gut microbial community is dominated by members of just two bacterial divisions, Bacteroidetes and Firmicutes, and one

member of Archaea, *Methanobrevibacter smithii* (Eckburg P.B. *et al.*, 2005). In contrast to the low levels of deep diversity, the microbial diversity in the mammalian gut exhibits high levels of variation at the level of species and strains.

In addition, the microbiota of the human intestine is quantitative and qualitatively different in the various compartements of the gastrointestinal tract (Figure 2) (Tiihonen K. *et al.*, 2010; Savage D.C., 1977; Berg R.D., 1996). On these differences a horizontal stratification is inserted with the presence of various microbial communities in the intestinal lumen, in the layer of mucus, in the crypts and directly adherent to the epithelial cells (Rozee K.R. *et al.*, 1982; Lee A., 1984).

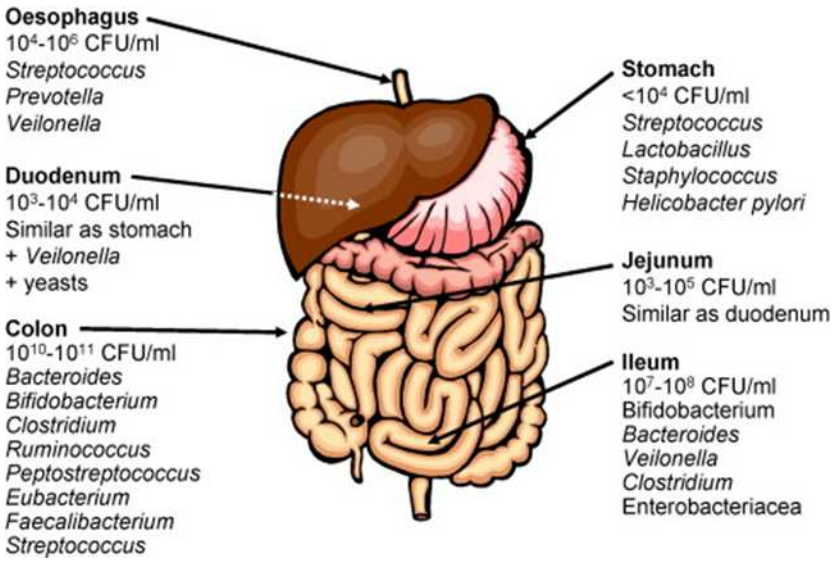


Figure 2. Common members of the intestinal microbiota in the different compartments of the gastrointestinal tract. (Tiihonen K. *Ageing Research Reviews.* 2010; 9: 107-16).

In numerical terms, esophagus and stomach show the lowest bacterial load. The normal esophageal microbiota is relatively simple and the preponderant cultivable bacteria are optional anaerobia that originates from the oral mucosa, like Streptococcus and Lactobacillus. Similarly the stomach, contains low levels of lactobacillus and other acid resistant microorganisms (10^1-10^2/ ml of content), that usually come from the oral mucosa. The microbiota load increases with the progression toward the ileum and colon that represents the gastrointestinal tract with the highest concentration of bacteria that show remarkable microbial difference [10^{11}-10^{12} / ml of luminal content, about 60% of the fecal mass (Guarner F. and Malagelada J.R., 2003)]. While in the small intestine optional anaerobial bacteria are present, in the colon the microbiota is largely made up by anaerobes.

The microbiota of the infant is seeded at birth and is initially undifferentiated across the various body habitats. A variety of factors—including method of delivery (vaginal versus Cesarian section), breast feeding, and weaning— influence the infant microbiota. For example, the microbiota of babies delivered vaginally are dominated by Lactobacillus, Prevotella, and Atopobium, whereas babies delivered by Cesarian section have a microbiota that more closely resembles that of the maternal skin community, with staphylococci being a dominant early member (Ravel J. *et al.*, 2011). The dominance of aerobic bacteria at birth is altered during peri- and postnatal development. The microbiota diversifies over the first few weeks of life to form a complex anaerobe-dominated microbial community (Mitsou E.K. *et al.*, 2008). However, within the second year of life the microbiota composition becomes more stable and similar to adult gut microbiota, although some studies have reported that adolescents have a higher abundance of bifidobacteria and clostridia than adults (Agans R. *et al.*, 2011). During old age a final set of age-related shift in the composition and function of microbiota is observed (Qi Y. *et al.*, 2012).

Functions of the microbiota

The intestinal microbiota has been demonstated to be involved in metabolic, trophic and protective functions (Nicholson J.K. *et al.*, 2005). In addition microbiota contributes to the development and function of:

- Metabolic activity and trophic functions

The most important metabolic activity of intestinal microbiota is represented by the fermentation of non digestible residues of the diet and/or of endogenous mucus produced by the epithelium. The host's mucus, in addition to provide protection against the adhesion and the invasion of pathogens, represents an important reservoir of glycans, mucin, glycosphingolipids, hyaluronic acid and heparin (Hooper L.V. *et al.*, 2002) and contribute to limit the potential damages caused from drastic changes in the availability of polysaccharides in the diet (Bäckhed F. *et al.*, 2005).

The intestinal bacteria draw the required energy through the metabolism of sugars and proteins (activity known with the term of fermentation). The modification of non digestible polysaccharides of the diet (cellulose, hemicellulose, pectin, non-digestible starch) is achieved with bacterial enzymes that modifies these latter substrates in volatile (carbonic anhydride, hydrogen, etc.) compounds and Short-Chain Fatty Acids (SCFA The synthesis of the fatty acids produces an acidification of the intestinal pH, that constitutes an efficient system of defense against pathogens microorganisms. The generation of SCFA, influences also the epithelial intestinal cells, facilitating their growth and differentiation. In fact, butyrate constitutes the principal energetic source of the epithelial cells of the colon (around 70% of the energetic intake). Acetate and propionate are, by contrast, able to regulate the glucose metabolism; their absorption induces a lower glycemic response after ingestion of oral glucose or a standard meal. The microorganisms of the colon also

develop a fundamental role in the synthesis of vitamins, as B12 and K and in the absorption of calcium, magnesium and iron.

- Protective function:

This function involves the inhibition of the pathogen flora. This is accomplished by the competition for the nutritional substrates and the adherence to epithelial cells (Bernet M.F. *et al.*, 1994). Germ-free animal, result more sensitive to the infections (Taguchi H. *et al.*, 2002).

- Functions on the development of the immune system of the host

The microbiota has also a pivotal role in the development of the immune mucosal system, both in its development and function; in germ-free mice the intestinal colonization is followed by an enhanced number of intraepithelial lymphocytes and of the lymphocytes infiltrating the lamina propria as well as by an increased number and size of the lymphoid follicles that show the presence of germinal centers containing immunoglobulin producing lymphocytes. The immune response towards the microorganisms relies on both innate and adaptive components as immunoglobulin secretion. The greatest part of the bacteria that are found in the human feces appear covered by specific IgA. The adaptive response of secretory IgA towards the intestinal microbiota allows the host to response to fluctuations in the bacteria concentration thus contributing to the mucosal homeostasis (Macpherson A.J. and Uhr T., 2004).

1.1.2 The intestinal barrier

The intestinal barrier is constituted by a single layer of epithelial cells that separates the intestinal lumen from the internal environment. The intestinal epithelium includes different types of cells: the enterocytes that have absorptive function, the goblet cells appointed to the production of mucus, the Paneth cells that have the ability to produce antimicrobial peptides and enteroendocrine cells. Altogether the intestinal epithelium extends to a surface of 400 m^2 covering anatomical structures organized so that to offer the maximum surface in the smaller possible (conniving valves, villi, microvilli) space. The integrity of the epithelial barrier is crucial in protecting the host from the microorganisms and from the alimentary antigens. The different described epithelial cells contribute to this function: the enterocytes migrate from the bottom of the crypts to constitute the epithelium of surface endowed with tight junctions constituted by trans-membrane proteins , claudin and occludin linked by non covalent bonds. Such junctions develop a sealing function forming around the cellular perimeter a continuous belt called zonula that limits the passage of water-soluble molecules; moreover they contribute to the maintenance of the polarity of the epithelial cells. The muciparous cells strengthen the epithelial barrier thanks to the production of

mucus that covers the whole intestinal surface. The mucins are the principals component of the mucus that covers the epithelial surface of the gastrointestinal tract. The mucins is a glycoprotein whose glucidic part, being highly absorbent, attracts a lot of water increasing the stringiness. The mucus is organized in a double layer with a denser layer to contact with the epithelial cells and one, less dense, directed toward the intestinal lumen. The microorganisms of the intestinal flora primarily occupy this layer reaching only in small measure the deep layer.

The Paneth cells of the small intestine and cells with analogous function in the colon are located on the bottom of the crypts and, due to their ability to produce antimicrobial pepetides, they limit the concentration of the bacteria penetrated in the deep layer of the mucus.

The integrity of the epithelial barrier and the intestinal homeostasis depends in great part on the continuous information interchange between the microbiota and the cells of the innate immunity with the contribution of the epithelial cells. To this purpose myeloid cells (dendritic cells, macrophage) as well as epithelial cells express surface receptors (Toll like receptor) and intracellular receptors (NOD - Nucleotide-binding-oligomerization domain) able to distinguish molecular specific determinants, conserved over the evolution, of microbial (PAMPs) origin. The microbiota / epithelial cells interaction triggers a physiological state of cellular activation with production of cytokine and chemokine and anti-apoptotic factors that stimulate the survival of the epithelial cells, their proliferation and the rapid reconstitution of the epithelial layer after the damage. The same receptors are also implicated in the production of antimicrobial peptides by the Paneth cells.

1.1.3 GALT

The lymphoid tissue associated to the intestine (Gut Associated Lymphoid Tissue, GALT), represents the wider lymphoid tissue of the whole organism, considered the enormous surface of the intestinal mucosa to which is in partnership (Figure 1). The lymphoid cells are either organized in follicles (Peyer's patches in the small intestine, lymphoid follicles in the colon) or dispersed in the lamina propria and in the layer of epithelial cells. The lymphoid structures (Peyer's patches, colon's lymphoid follicles) together with the mesenteric lymph nodes represent the sites of induction of the immune response. The dendritic cells once captured the antigens at the mucosa surface migrate into these sites in order to present the antigens to T lymphocytes. The T lymphocytes, once primed by the dendritic cells acquire the role of effectors of the immune response with different function (lymphocytes $CD4^{+}$Th -1, lymphocytes $CD4^{+}$Th -2, lymphocytes $CD4^{+}$Th -17, lymphocytes regulators). They then migrate in the circulatory stream after the acquisition, on the surface, of the expression of integrins ($\alpha4\beta7$, $\alpha E\beta7$) that allow their recognition

by the lymphoid intestinal tissue where, they migrate back to perform their effector functions in the lamina propria (lymphocytes α4β7+) and in the epithelium (lymphocytes αEβ7+).

Dendritic cells:

Dendritic cells migrate from the tissues to the secondary lymphoid organs in order to present the antigen to the T cells. Although at the beginning they were considered as activators of the immune system in response to external stimuli (infectious agents), recently they have been recognized to play a fundamental role in the induction and maintenance of host tolerance. Tolerance represents the establishment of an immune-mediate process that prevents the inflammatory response toward endogenous antigens, harmless food-derived antigens or antigens associated to the microflora. The circulating precursors of the dendritic cells are distributed in the peripheral tissue and in the lymphoid organs, where they differentiate in two types of dendritic cells characterized by different functions: the plasmacytoid dendritic cells and the conventional dendritic cells. Dendritic cells are in two functionally different conditions: naïve and mature dendritic cells. Naive dendritic cells are present in the peripheral tissue where they have mainly phagocytic functions, whereas mature dendritic cells are found in the lymphoid organs and they are specialized in the antigen presentation and characterized by the expression of surface of costimulatory molecules that are mandatory for the activation of the T cells. Mature dendritic cells derive from the naive ones after a process of maturation triggered by inflammatory stimuli and that promotes the migration of the dendritic cells in the lymph nodes. Under homeostatic condition, nevertheless, there is a low but constant migration of naive dendritic/semimature cells from the peripheral tissue to the draining lymph nodes. These cells are probably involved in the induction of the tolerance to self-antigens . Dendritic cells isolated from the intestinal tissue are endowed with particular functional features including the ability to induce peripheral regulatory $Foxp3^+$cells, the differentiation of Th-17 cells and the Ig-A switch in the B cells. These functions are associated to different types of dendritic cells that are distinguishable for the presence of different surface antigens. At least three different functionally types of dendritic cells have been described. $CD103^+$ dendritic cells expressing high-level of $CD11c^+$ able to promote, under the influence of retinoic acid and TGF -β the development of regulatory cells to limit the inflammatory response response toward the microbial antigens. Dendritic cells expressing high-level of $CD11b^+$ and TLR5 able to induce the differentiation of Th-17 cells and a third type characterized by the expression of the CD70 and CX3CR1 able to response to the ATP of bacterial origin inducing the differentiation of Th-17 cells. Dendritic cells able to induce Th-17 seem to be e also able to favor the IgA switch in the B cells. Since the intestinal dendritic cells probably derive from the circulating monocytes migrated inside the tissue, the different functions of the dendritic cells strictly derive from the interaction with the microenvironment. Different cellular types contribute directly or indirectly to the mucosal conditioning of the dendritic cells: the epithelial cells through the production of TSLP able to

inhibit the synthesis of IL-12 and the production of TGF-β and retinoic acid able to direct the dendritic cell toward the induction of regulatory cells. Stromal cells through the production of TGF-β that inhibit the production of cytokine inflammatory by resident macrophages. These conditioned macrophages limit the ability of the dendritic cells to induce Th -17 cells.

T lymphocytes:

The $CD4^+$ T lymphocytes are classically classified in T helper (Th-1, Th-2, Th -17) and T regulatory cells. The Th-1 lymphocytes are inflammatory lymphocytes that releases IFN-γ and are involved in the immunity against the pathogenic intracellular agents, while the Th-2 lymphocytes are mainly involved in the B cells response providing growth factors as IL -4. The Th-17 lymphocytes produce a range of cytokine as IL-17A, IL-17F, IL-21, IL-22, IL-26 involved in the activation of the neutrophils and in the immune response against extracellular bacteria and fungi. Under pathological conditions, as in inflammatory bowel disease, autoimmune diseases or in the allergies, T lymphocytes are responsible for the maintenance of the chronic inflammation and for its polarization. For example, inflammatory bowel disease, are associated with Th-1 polarization in the case of Crohn's disease and to Th-2 polarization in the case of ulcerative colitis. The Th-17 response , that is present in both pathologies, seems to have a protective role, but under pathological conditions as in the case of autoimmune diseases, seems instead to develop a key role in the occurence of chronic inflammation. The T regulatory lymphocytes suppress the function of the T effector cells and they are therefore essential to counteract the inflammatory responses (Zhu J. and Paul W.E., 2010).

• T helper cells

T helper cells 1 (Th1) are characterized by the production of interferon -γ (IFN -γ) sustained by IL-12 and IL-23 produced by the denditric cells and they are important for immune responses towards intracellular pathogens (virus and bacteria). The fundamental transcription factors required for the Th-1 differentiation and production of cytokine are T-bet and STAT. Particularly IFN - γ induces the activation of STAT1, IL-12 induces the activation of STAT4 and these two transcription factors upregulate T-bet, that plays a fundamental role in the Th -1 differentiation. T-bet is a negative regulator of GATA-3, fundamental transcription factor in the Th2 differentiation. IFN-γ stimulates the anti microbial activity of the phagocytes favoring the clearance of the phagocytosed microorganisms; in addition it stimulates the production of IgG able to opsonise the bacteria and to activate the complement; these two mechanisms favor the phagocytosis and subsequent clearance of bacteria. IL-2 produced by the same Th1 acts as autocrine growth factor and together with the IFN-γ stimulates the proliferation and differentiation of the cytotoxic CD8+ T lymphocytes that kill the infected cells. Finally IFN-γ amplifies the differentiation of the CD4 T cells in Th1 inducing the

activation of the macrophages with production of IL-12. T helper 2 cells (Th2) are characterized by the production of IL-4, IL-5, IL-13 and IL-25 and they play a critical role against the helminth infections and allergic reactions. The TH-2 differentiation of T lymphocytes depends on the presence of IL-4 that activates STAT 6, a transcription factor that induces the expression of GATA-3, a transcription factor distinctive of Th2 cells. GATA -3 inhibits the intracellular signaling of STAT4, that is required for Th1 differentiation. The principal Th2 effector functions are represented by activation and expansion of eosinophils and mast cells and IgE production. Such functions are mediated by IL-4, IL-5 and IL -13 production.

From above, it is appreciable that the functional polarization of T cells is not only influenced by the type of antigen and its concentration, but also by the presence of particular co-stimulation signals, by the prevailing cytokines in the environment and by the transcription factors that, through a positive or negative feedback activate and inhibit a specific lineage program. In this context it is useful to underline that recent studies have shown that IL-25, a family member of the IL-17, produced by T cells , but also by mast cells, alveolar macrophages, eosinophils, and basophils, pulmonary epithelial cells and intestinal epithelial cells, starts, promotes and expands the Th-2 mediate response favoring the establishment of some allergic diseases (Caruso R. *et al.*, 2011) and it inhibits the Th-17 response in animal models of autoimmune diseases as multiple sclerosis and diabetes.

The T 17 helper cells (Th-17) have been recently described as lineage with independent differentiation. They are involved in the infections by extracellular bacteria and fungi through the production of IL-17A, IL-17F and IL -22. They differentiate in presence of TGF -β and IL-6, which activate STAT3, that positively regulate the transcription factor RORγt, a fundamental factor for Th-17 differentiation. IL-23 represents, together with IL-21, an important factor for their survival and for their expansion. A peculiar aspect of the Th-17 cells is their plasticity. In literature there are evidences about the possibility that, in addition to their differentiation from naive cells as a consequence of the above described stimuli, they can derive from regulatory $Foxp3^+$ T cells in the presence of high-level of IL-6. This inflammatory cytokine would inhibit the expression of Foxp3 favoring the expression of RORγt. Recent data point out that also IL-1 could favor this passage. Finally, Th-17 cells, in presence of high-level of IL-23 and reduced levels of TGF-β can express IFN-γ maintaining the expression of IL-17 that can be later on definitely lost. These Th-17 derived IFN-γ cells producing cells are called simil Th -1. The final step of conversion (IFN-γ+ IL-17-) is inhibited by $CD4^+CD25^+Foxp3^+$ regulatory cells favoring the expansion of IL-17+IFN-γ+ T cells (Abdulahad W.H. *et al.*, 2011;. Morrison P.J. *et al.*, 2011).

• *Regulatory cells*

Regulatory cells are able to inhibit the response and the proliferation of the effector T cells. Two subpopulations of cells can be identified: natural $CD4^+$ $CD25^+$ (nTreg) regulatory T cells and peripheral regulatory T cells. The natural regulatory cells are originated in the thymus and constitutively express on their cellular membrane the CD25 (chain of the receptor of the IL-2 (IL-2R)), the CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) and the GITR (glucocorticoid-induced TNF receptor family-related gene). A intracellular marker that is essential for the development and the function of regulatory cells is the transcription factor Foxp3 (X-linked, forkhead / winged helix transcription factor) (Hori S. and Sakaguchi S., 2004). Absence of Foxp3 is associated to a morbid condition linked to the chromosome X, called "IPEX", characterized by immune dysregulation, polyendocrinopathies, enteropathies. Foxp3 represses the promoters of the genes involved the inflammation (Izcue A. and Powrie F., 2007).

Peripheral regulatory cells are classically divided into 3 groups: inducible $Foxp3^+$ cells (iTreg), regulatory T cells (Tr1) and the Th3 cells. To these 3 groups, a fourth group of cells has recently been added: the cells that express on their surface the TGF -β linked to its latency peptide called Latency-associated-Peptide (LAP+) T cells. The difference among the different groups of regulatory cells relies on the expression of Foxp3 that seems to characterize the iTregs alone and on their prevalent mechanism of suppression: cytokine-mediated, respectively IL-10 and TGF-β mediated for the Tr1 and Th-3 cells, contact-mediated for the iTreg and probably for the LAP+ cells. Other studies have however shown that the production of IL-10 and TGF-β is essential for the suppressive function of the $Foxp3^+$ cells and that the expression of cell surface LAP seem to characterize also the activated Foxp3 cells. At present the classification of regulatory T cells into inducible $Foxp3^+$ cells (iTreg) and $Foxp3^-$ (Tr1, Th-3) seems to be the most appropriate. Inasmuch it is becoming more and more evident that $Foxp3^-LAP^+$ cell population can represent a separate group of regulatory cells, nevertheless its relationship with the Tr1 and Th-3 cells it is still object of study.

The number and/or function alterations of the various types of regulatory cells are involved in several chronic diseases, such as autoimmune diseases, chronic inflammatory bowel diseases and allergies.

One of the major issues being debated is the pleiotropic nature of TGF-β. On the one hand, it regulates the homeostasis of nTregs and perpetrates the suppression of the effector cells (inhibiting Th1 and Th2), and, on the other hand, as previously pointed out, it succeeds, together with IL-6, in inducing the Th-17 differentiation. A few studies have shown that murine CD4 (+) CD25 (+) T cells stimulated *in vitro* with antiCD3/antiCD28 antibodies may perform their suppressing action on the effector cells using TGF-β as effector mechanism; TGF-β is not in its secreted form but in the form

of a factor linked to the cell membrane and succeeds in mediating an interaction with the receptors present in the target cells. TGF-β is synthesized in the form of precursor that is cleaved intracellularly by endopeptidase. The proteolysis yields two products that assemble into a dimer. One is latency-associated peptide (LAP), and the other is mature TGF-β. The presence of LAP facilitates the transit to the cellular surface and keeps TGF-β biologically inactive and prevents interactions of TGF-β with receptors. The LAP-associated mature TGF-β is referred to as "latent TGF-β"; it is secreted as precursor and converted in the biologically active form in the extracellular space through the removal of LAP. The mechanisms that lead to the removal of LAP are not fully known, even though it is assumed that the thrombospondin present on the macrophages should be able to affect the LAP-TGF-β complex (Khalil N., 1999), releasing the active form of cytokine.

Host/microbiota recognition and interaction

The various host/microbiota interactions described above occur through the recognition of bacterial/viral/fungal molecules by specific receptors arranged on both the cellular surface and inside the cytoplasm.

1.1.4 Toll-like receptors

Toll-like receptors are a family of pattern recognition receptors (PRR) that have not changed in the course of evolution. These receptors are expressed by various cells involved in the innate responses to pathogens, recognizing a few molecular profiles expressed by the pathogen-associated molecular patterns (PAMPs) (Medzhitov R. and Janeway C.A. Jr., 1997). As a matter of fact, even non-pathogenic bacteria, such as those of the intestinal microflora, use the same structures for their recognition by the epithelial cells and by the cells of the innate and acquired immunity of the host.

TLRs have the type I structure of transmembrane proteins with multiple extracellular cysteine and leucine-rich repeats (LRRs) and exhibit a single Toll/interleukin-1 (IL-1) receptor (TIR) intracellular domain which is similar to the intracellular receptor domain of the IL -1 family.

There are 13 TLRs in human and mice that share a similar structure and an ancestral gene. An important characteristic of the signaling system is that TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and a set of TLRs sensitive to nucleic acids -TLR3, TLR7, TLR8 and TLR9 - are intracellular (Figure 3).

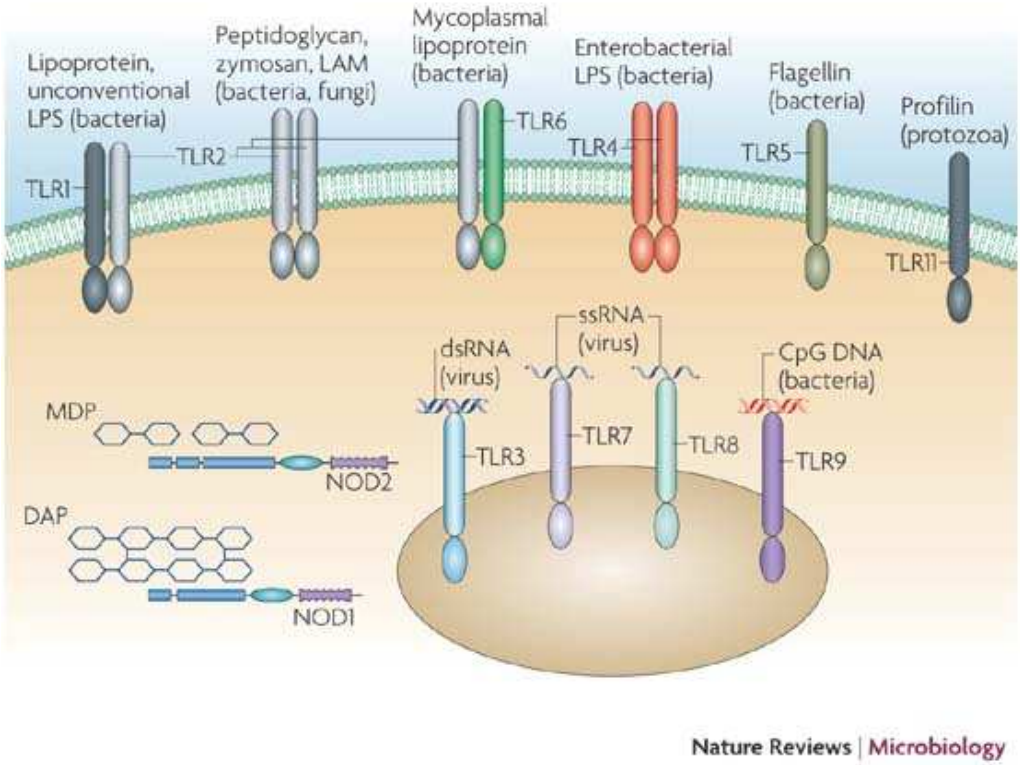


Figure 3. TLRs localization. (Kaufmann S.H., *Nature Reviews Microbiology* 2007; 5: 491-504)

The lipopeptides and the other components of the Gram-positive bacteria cells activate TLR2 together with TLR1 or TLR6; LPS activates TLR4, flagellin activates TLR5, Poly I: C (a double-stranded RNA (dsRNA) activates TLR3; unmethylated DNA and CpG oligodeoxynucleotides (or CpG ODN)) activate TLR9, while single-stranded RNA and a few drugs activate TLR7.

The ligands for TLR8 and TLR10 (present only in humans) and for TLR11-13 (present only in mice) are not known (Figure 4).

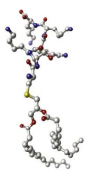
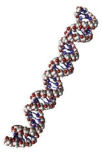
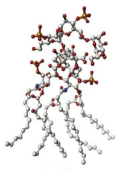
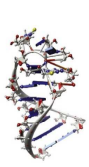
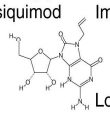
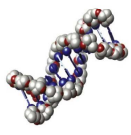
Receptor	TLR2/1 or 2/6	TLR3	TLR4	TLR5	TLR7	TLR9
Ligands	Lipopeptides	Poly I:C, dsRNA	LPS	Flagellin	ssRNA, resiquimod, imiquimod, loxoribine	Unmethylated DNA, CpG-DNA
Source	Gram-positive bacteria, fungi	Viruses	Gram-negative bacteria	Bacterial flagellum	Viruses	Bacteria
Examples	 Pam_2CSK_4	 dsRNA	 LPS	 Flagellin	 ssRNA  Resiquimod  Imiquimod  Loxoribine	 CpG-DNA

Figure 4. TLR ligands and interactions with receptors. (Moresco E.M., *Current Biology* 2011; 21: R488-93).

The TLRs are expressed by macrophages and neutrophils, as well as by dendritic cells and specialized phagocytes that are to stimulate the immune responses by activating the T-lymphocytes.

Many “non-immune” cell types, including epithelial cells, neurons, astrocytes, and fibroblasts, also express TLRs and respond to their activation.

TLR signaling depends critically on four adaptor proteins (Figure 5):

- MyD88 (Myeloid differentiation primary response gene (88));
- TIRAP (also called MAL);
- TICAM1 (also called TRIF);
- TICAM2 (also called TRAM) that directly bind to activated TLRs.

There are two signaling pathways: the MyD88-dependent and the TRIF-dependent pathways.

All TLRs (except TLR3), utilize a MyD88-dependent pathway after the dimerization of the TLR receptor. As far as the signal transmitted by TLR4 is concerned, a MyD88-dependent transmission pathway is known to activate the cytokine production and a MyD88-independent pathway is known to activate the type I interferon system.

Once activeted, MyD88 recruits IRAK 4, IRAK1 and IRAK2. IRAK kinases then phosphorylate and activate the protein TRAF6, which in turn polyubiquinates the protein TAK1 in order to facilitate binding to IKKβ. On binding, TAK1 phosphorylates IKKβ, which then phosphorylates Iκβ causing its degradation and allowing NF-κB to diffuse into the cell nucleus and activate transcription. TAK1 may also trigger MAP (mitogen-activated protein) kinases with activation of AP1 and CREB (cyclic AMP response element-binding protein) (Kawai T. and Akira S., 2010). Signal transduction from TLRs induces the expression of numerous genes required for the inflammatory response, including inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, IL-12 and IL-18), chemokines, antimicrobial molecules (such as hydrolytic enzymes, peptides, proteases), and costimulatory molecules important for adaptive immune activation.

The TLR3 transmission pathway involves the recruitment of the TRIF adaptor protein and TRIF activates the TBK1 and RIP1 kinases; TRIF/TBK1 signaling phosphorylates IRF3 allowing its translation into the cell nucleus and the production of interferons type I (IFN-β). The activation of TLR7 and TLR9 activates a MyD88-dependent signal transmission that may also activate the IRF7 (interferon regulatory factor 7) transcription factor with the activation of interferons type I (IFN-α) that are powerful anti-viral cytokines.

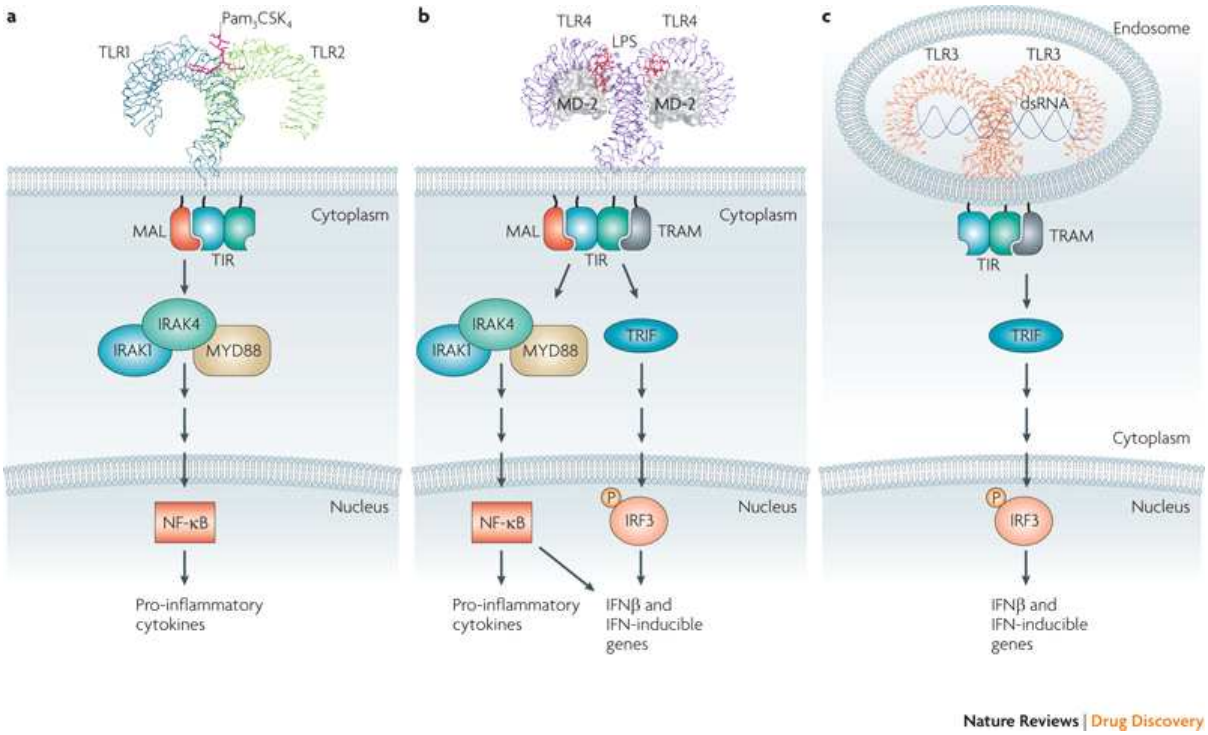


Figure 5. Signaling TLRs. (Hennessy E.J., *Nature Reviews Drug Discovery* 2010; 9: 293-307)

TLR2 recognizes peptidoglycan (PGN), the component of the bacterial cell-wall of several Gram-positive pathogenic and commensal bacterial strains.

Studies of TLR2-deficient mice or cells derived from these animals have shown that TLR2 is necessary for the response to PGN, the mycoplasma lipopeptide that activates the macrophage-activating lipopeptide-2 (MALP-2) (Takeuchi O. *et al.*, 1999, 2000, 2001), as well as for the response to Lipoarabinomannan, a glycolipid associated with *Mycobacterium tuberculosis* (Jones B.V. *et al.*, 2001). Besides, even the recognition of the lipoteichoic acids (lipidic components of the cytoplasmic membrane associated with the teichoic acids of the Gram-positive wall) and *Listeria monocytogenes* (a Gram-positive organism) would seem to involve TLR2.

According to a few studies, the capacity of TLR2 to bind such a variety of ligands is founded on its ability to form heterodimers with other TLRs, above all TLR6 and TLR1 (Ozinsky A. *et al.*, 2000; Takeuchi O. *et al.*, 2001).

The TLR4 receptor distinguishes both bacterial PAMPs and viral PAMPs. However, the best represented PAMP is LPS, an important element of the external membrane of Gram-negative bacteria.

The LPS-TLR4 bond is mediated by CD14 and MD-2 proteins strengthened by LBP (LPS-Binding Protein), a soluble protein present in the plasma. LPS induces several immunostimulant responses, including the production of proinflammatory cytokines as IL-12 and inflammatory chemokines.

Following the interaction with bacteria and/or viruses, antigen-presenting cells (APCs), macrophagic cells and dendritic cells, which express TLRs on their surface, bind PAMPs and begin a signaling series that stimulates the host's defenses through the induction of ROS (Reactive

Oxygen Species) and nitrogen.

Later, the activation of these cells leads to the production of proinflammatory cytokines that, in turn, upregulate costimulatory molecules, stimulating the maturation of dendritic cells and their migration in the lymph-nodes.

The cell migration in both mice and humans is mediated by the chemokine receptors expressed by the dendritic cells following the stimulation of TLRs (Rescigno M. *et al.*, 1999; Sallusto F. *et al.*, 1999).

The dendritic cells, getting to the secondary lymphoid organs, stimulate the T cells through the presentation of peptides associated with MHC molecules, linked with a secondary signal transmitted by costimulatory molecules as CD80/86.

MHC are proteins codified by a multiplicity of genes called major histocompatibility complex. They are entrusted with the task of binding the peptidic fragments produced by the degradation of foreign protein antigens inside the target cell and transporting them to the surface. Hence, the T response, mediated by the Th-1 or Th-2 polarization, is induced at the lymph node level.

Based on the foregoing, it seems reasonable to conclude by saying that TLRs are indispensable not only in the initial infection phase, when they contribute to the innate immune response, but also in the passage from innate immunity to adaptive response, when they condition the response of the dendritic cells responsible for educating T cells to the antigenic recognition and for the response guidance (Werling D. and Jungi T.W., 2003).

1.1.5 NOD2

NOD2 (nucleotide-binding oligomerization domain-containing protein 2) belongs to the NLR (NOD-like receptors) family of pathogens/microbe-associated molecular patterns (Strober W. *et al.*, 2006; Inohara N. *et al.*, 2005).

The *NOD2/CARD15* gene (Figure 6), found on chromosome 16 in the pericentromeric region 16q, is the most important gene involved in the susceptibility to Crohn's disease (CD).

NOD2 encodes a protein of 1,040 amino acids that is expressed in macrophages, monocytes, dendritic cells, epithelial intestine cells and in the Paneth cells located in the terminal ileum.

The protein comprises 2 N-terminal CARD domains, a central oligomerization domain (NOD), and a C-terminal stretch of leucine-rich repeats (LRRs). The CARD (Caspase Activation Recruitment Domain) domains are responsible for the bond with other proteins with similar domains.

The NOD (Nucleotide Oligomerization Domain) mediates the oligomerization of the proteins comprising the CARD domains, allowing the activation of the NF-kB transcription factor.

Just like the LRR (Leucine-Rich-Repeat) of the toll-like receptors, the LRR domain mediates the response to the muramyl dipeptide component (MDP) of peptidoglycan (PGN) of Gram-positive and Gram-negative bacteria. The NOD2 protein acts as an intracellular sensor in the innate immune response to Gram-negative and Gram-positive bacteria.

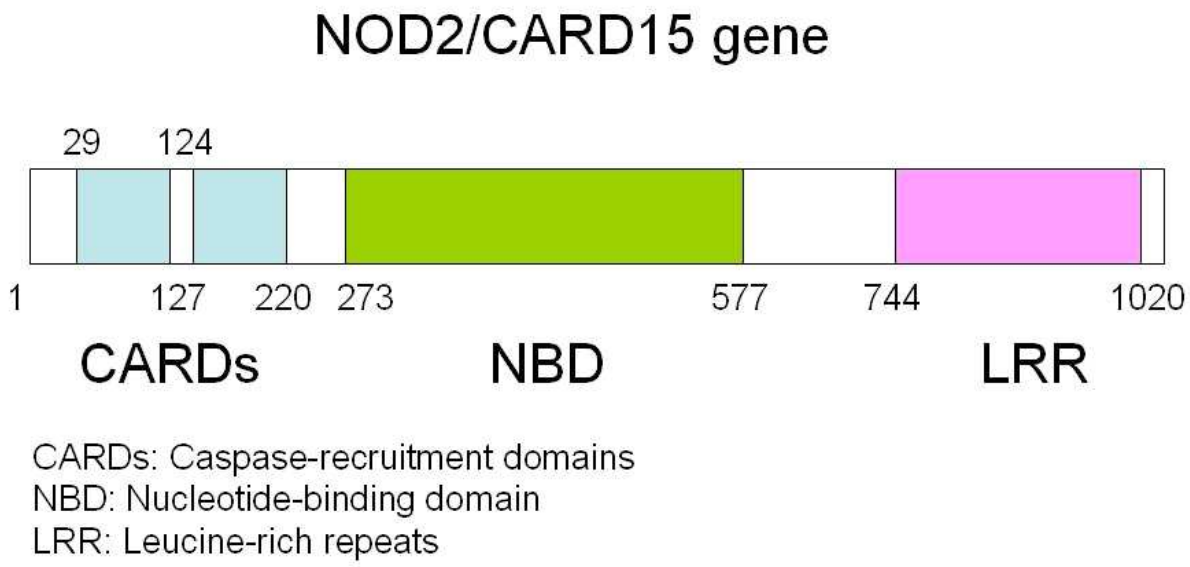


Figure 6. *NOD2/CARD15* gene structure.

The MDP stimulation activates NOD2 through an initial conformational change in the structure, followed by oligomerization and exposure to the CARD domain (Figure 7). In its turn, the latter binds to a downstream adapter molecule known as RICK (or RIP2) through a CARD-CARD interaction. The final result is the activation of MAP-kinase (mitogen-activated protein kinase) and NF-kB (nuclear factor-kB) that lead to the synthesis of various proinflammatory cytokines and/or chemokines (Strober W. *et al.*, 2006).

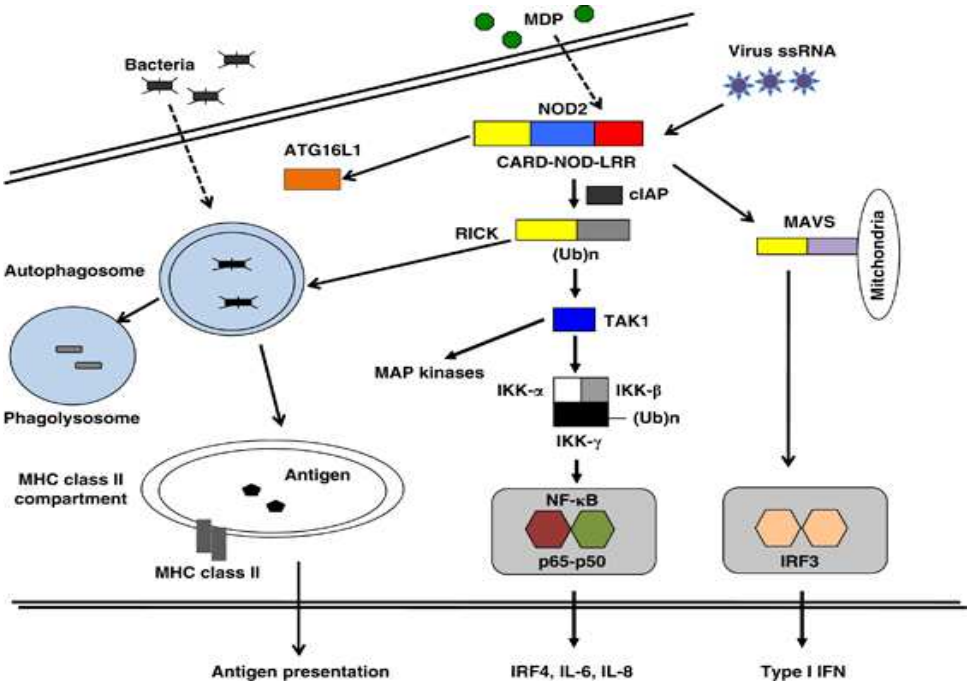


Figure 7. NOD2 interactions. (Strober W. *Mucosal Immunol.* 2011; 4: 484-495)

The MDP-induced NOD2 signaling acts also through a RICK-independent mechanism. NOD2 responds *in vitro* to viral ssRNA or *in vivo* to viral infection (such as respiratory syncytial virus) with IRF3 activation (Sabbah A. *et al.*, 2009). This response involves the binding of ssRNA to NOD2, followed by translocation of NOD2 to the mitochondria and interaction with MAVS (mitochondria anti-virus signaling protein), a mitochondrial adaptor molecule. Finally, the MAVS leads to activation of downstream signaling molecules that lead, in their turn, to the production of interferon-β and NF-κB activation. Overall, this NOD2 activation pathway is substantially different from the MDP-induced pathway in that it involves a different ligand and a distinct downstream signaling pathway.

Studies conducted on mice and humans have shown that the NOD2 stimulation of MDP in cells translates into variable responses that, in most cases, are lower than the responses obtained with TLRs stimulation (Watanabe T. *et al.*, 2004). Although NOD2 stimulation itself results in weak responses, such stimulation has a considerable capacity to increase TLR responses. Such increase may result from the fact that TLR stimulation upregulates NOD2 expression, and combined stimulation of cells through upregulated NOD2 and a TLR has a synergistic effect similar to that seen for other combinations of TLR stimulants (Fritz J.H. *et al.*, 2005; Hirata N. *et al.*, 2008).

NOD2 responses can complement or enhance innate immune response mediated by TLRs or other innate response elements and this occurs by virtue of its position as an intracellular response factor that, therefore, is involved in the neutralization of pathogens or in response to intracytoplasmic danger signals. NOD2 responses can conceivably provide host defense function in areas of the body where TLR expression is reduced because TLR function has been downregulated by constant exposure to TLR ligands. In fact, a tolerance-oriented down-modulation mechanism is activated in these areas. This is what happens in the gastrointestinal (GI) tract where the commensal microflora provide a source of TLR ligands that render gut epithelial cells or antigen-presenting cells tolerant to TLR ligands (Strober W. *et al.*, 2006). Another mechanism by which NOD2 contributes to host defense is by binding to the inflammasome protein NLRP1; the complex binds pro-caspase I through the CARD domains of NOD2 and/or the NLRP1, leading to the activation of caspase I, which then acts on pro-IL-1β or pro-IL-18 to form mature forms of these cytokines that can then be secreted (Hsu L.C. *et al.*, 2008).

A new NOD2 functional activity is represented by its implication in autophagy.

Autophagy is a complex intracellular protein degradation mechanism by which the cell forms double-membrane vacuoles (autophagosome) that ultimately fuse with lysosomes to eliminate proteins arising from cellular stress responses (Mizushima N. *et al.*, 2008). This mechanism also involves the breakdown of ingested pathogenic bacteria providing antigens for antigen presentation in the immune response useful for host defense.

Studies have shown that the NOD2 protein binds to an autophagic protein, ATG16L1, and co-localizes with NOD2 at the plasma membrane level, thus facilitating the formation of an

autophagosome around the invading bacteria (Travassos L.H. *et al.*, 2010).

NOD2 has an unequivocal function as a host defense factor not only in relation to bacteria which express peptidoglycan, and thus MDP, but also in relation to viruses and parasites.

NOD2 intervenes in the intestine in defense of the host through the involvement of specialized gut cells in the intestinal crypts called Paneth cells, which produce considerable amounts of α-defensins, which are NOD2-dependent anti-bacterial peptides.

Finally, the NOD2 prominence also derives from the fact that polymorphisms in the LRR region of *CARD15* are genetic risk factors for the occurrence of such diseases as Crohn's disease, graft vs. host diseases (GvHD), and allogeneic bone marrow transplantation. In addition, the mutations in the NOD region of *CARD15* are causative of Blau's syndrome, a granulomatous inflammation affecting the eyes, skin, and joints.

1.2 INFLAMMATORY BOWEL DISEASES (IBD)

The inflammatory bowel diseases (IBD) comprise Crohn's disease (CD) and ulcerative colitis (UC) (Baumgart D.C. and Sandborn W.J., 2007).

Ulcerative colitis is characterized by inflammation that is restricted to the colon: it starts in the rectum and extends proximally in a continuous manner and often affects the periappendiceal region. On the other hand, Crohn's disease affects any part of the gastrointestinal tract - mostly the terminal ileum - in a non-continuous manner and, unlike ulcerative colitis; it is generally associated with complications as stenoses, abscesses and fistulae. From a histological point of view, ulcerative colitis features an inflammation that affects for the most part the superficial layers of the mucosa (epithelium and lamina propria) with presence of epithelial damage and formation of cryptic abscesses. Crohn's disease is instead characterized by a transmural inflammation affecting all the layers of the mucosa up to the serosa, with presence in about 15% of all cases of non-caseificant granulomas. In North America, the IBDs have an incidence of 2.2 to 14.3 cases per 100,000 individuals per year with respect to UC and 3.1 to 14.6 cases individuals per year with respect to CD, while in Europe they have an incidence of 8.7 to 11.8 cases per 100,000 individuals per year (UC) and 3.9 to 7.0 cases per 100,000 individuals per year (CD), respectively. UC is more frequent than CD, regardless of the geographic area being considered, with an estimated number of 780,000 individuals affected by UC and 630,000 which suffer from CD in the United States (Loftus E.V. Jr., 2004).

The geographic variability observed in the incidence, side by side with economic growth and the increase in the per capita income, together with the prevalence rate change observed in a few ethnic groups after their migration to other geographic areas, represent a further support to the theory that environmental factors and lifestyles play a role in the onset of the disease (Loftus E.V. Jr., 2004).

The most creditable pathogenic hypothesis assumes that the onset of these diseases, in genetically

predisposed individuals, is based on an altered immune response to one's bacterial flora. In particular, environmental factors would seem to stress genetically determined variations of the immune response responsible for an altered control of the response to the microbiota. At the same time, the action of environmental factors or the subsequent alterations of the immune response to the microbiota seems to favor the emergence of potentially pathogenic strains (the so-called pathobionts) that would contribute to the chronicization of the inflammation. All the aforementioned components, microbiota, environmental factors, immune response and genetic susceptibility contribute with mutual interactions to the onset of a clinically manifest disease (Figure 8).

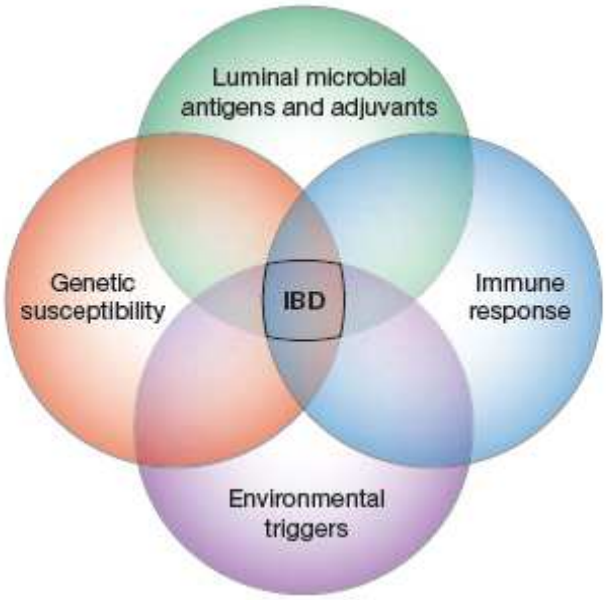


Figure 8. Interaction of various factors that contribute to IBD in a genetically susceptible host organism. (Sartor R.B., *Nat Clin Pract Gastroenterol Hepatol* 2006; 3: 390-407)

Genetic alterations:

Even though the precise etiology of IBD is so far still not clear, genetic analyses of familial aggregation and concordance studies of monozygotic twins have highlighted that genetic determinants play a role in both the penetrance of the disease and its heterogeneity. Linkage and familial association studies have allowed the identification of a variety of loci responsible for IBD susceptibility.

Quite recently, following the introduction of Genome-wide association studies for the identification of single nucleotide polymorphisms (SNPs) associated with a greater frequency of the onset of certain diseases, numerous variations associated with a greater risk of developing Crohn's disease and ulcerative colitis were identified in patients suffering from IBD. The genes that are involved range from those implicated in the intestinal permeability to those concerning the immune response. The identification of single polymorphisms in the *NOD2* gene has represented the first recognized risk factor for Crohn's disease.

Nowadays, the *CARD15/NOD2* gene is viewed as the main gene involved in CD. Considering the

gene variants identified in the *NOD2* gene, three are associated with the susceptibility to CD and are present in about 40% of patients. The *NOD2* gene is formed by 11 constant exons and a twelfth alternative exon in region 5. *NOD2* encodes a protein of 1,040 amino acids containing various functional domains. Over 30 mutations affecting the entire *NOD2* gene have been identified. Out of these 30 genetic variants, three have proved to be independently associated with the susceptibility to CD and represent 82% of the chromosomal mutations.

These three allelic variants are:

- R702W (C2104T), positioned on exon 4;
- G908R (G2722C), positioned on exon 8;
- 1007fs (3020insC), positioned on exon 11.

The first two mutations may be considered non-conservative missense mutations. They have also been identified as disease-causing mutations, even though they do not induce structure modifications as the 3020insC variant does. Besides, 27 additional minor and rare mutations have been classified as mutations that could cause diseases (DCMs). The Leul007fiC (3020InsC) mutation determines the formation of a truncated protein lacking 33 distal amino acids in the LRR domain that entails a loss of the functional activity of the protein.

The other mutations are 2104 T~C, which causes the replacement of arginine 702 by a residual of tryptophan (R702W), and 2722 G~C, which causes the replacement of a glycine by an arginine at position 908 (G908R). These variants modify the proteinic composition and, therefore, interfere with the binding to MDP.

Microbiota Alterations

A huge number of micro-organisms are known to colonize the gut and to form complex communities or microbiota. The host-microbiota associations usually evolve maintaining a good relationship. Bacteria provide the gastrointestinal tract with nutritive substances and carry on the function of preventing the colonization by pathogenic opportunists. Furthermore, they contribute to the anatomic development and functioning of the mucosal immune system (Duerkop B.A *et al.*, 2009). In adults, the “normal” microbiota comprises not only bacterial species that support anti-inflammatory responses but also micro-organisms that, under special conditions, can cause inflammation (Round J.R. and Mazmanian S.K., 2010). Therefore, microbiota has the potential to evoke both pro and anti-inflammatory responses and the composition of the bacterial communities in the intestine is connected with the perfect operation of the immune system (Round J.R. and Mazmanian S.K., 2009). Both a decrease in the phyla *Firmicutes* and *Bacteroidetes* and a reduction of the member diversity were observed in patients with inflammatory bowel diseases (Frank D.N. *et al.*, 2007). Recently, a reduction of the bacterium *Faecalibacterium prausnitzii*, belonging to phyla *Firmicutes*, was associated with an early post-operative recurrence in patients with CD (Sokol H. *et al.*, 2008). Other studies have reported that a subset of people with Crohn’s disease harbor a strain

of potentially invasive *Escherichia coli* in their small intestine (Pineton de Chambrun G. *et al.*, 2008).

An altered balance of beneficial versus aggressive microbial species could lead to a proinflammatory luminal milieu that drives chronic intestinal inflammation in a susceptible host. Numerous studies have implicated several commensal organisms, such as *E. coli, Bacteroides, Enterococcus* and *Klebsiella* species, in the pathogenesis of human IBD. By contrast, various *Lactobacillus* and *Bifidobacterium* species have predominantly protective effects and have been used therapeutically as probiotics (Sartor R.B., 2004). Several groups showed alterations of the microbial luminal mucosally-adherent flora in patients with Crohn disease, ulcerative colitis and pouchitis (Swidsinski A. *et al.*, 2002; Sartor R.B., 2004).

A fundamental and still unsolved problem is whether dysbiosis is a factor of primary or secondary predisposition for IBDs, since it may be related to other defects or compounded by them. Recent studies have shown that dysbiosis is affected by both the genotype of the host, such as the presence of alleles of susceptibilities (for instance NOD2 or ATG16L1), and the IBD phenotype (a more significant difference is reported in patients with ileal Crohn's disease with respect to the controls). It should be considered that major commensal organisms belonging to the Clostridiales order, such as *Faecalibacterium* and *Roseburia*, appeared to be significantly reduced in patients with ileal Crohn's disease (Frank D.N. *et al.*, 2011; Willing B.P. *et al.*, 2010). These genii are considerable sources of short chain fatty acids, such as butyrate, which have proved to play a protective role in the murine models of colitis. Furthermore, Clostridium Clusters IV (including *Faecalibacterium*) and XIV were recently shown to induce the accumulation of Treg FOXP3+ cells in murine colon (Atarashi K. *et al.*, 2011).

Dietary components can alter the composition and virulence of enteric commensal bacteria, providing a potential explanation for the increase in the incidence of IBD in Western countries in the second half of the twentieth century and, more recently, in Eastern countries, as they adopt Western dietary practices. The dietary components can achieve opposite effects. For instance, short-chain fatty acids, especially butyrate, are the preferred metabolic substrates of colonocytes, and can positively affect the mucosal barrier. Iron stimulates growth and virulence of intracellular bacteria, whereas aluminum is an adjuvant for bacterial stimulation of immune responses (Perl D.P. *et al.*, 2004).

Epithelial barrier and immune response alterations

It is believed that a few defects affecting the intestinal barrier, such as an increase in the intestinal permeability and/or a defective repair of epithelial cell after transitory damages, may induce the onset of IBDs by favoring a greater passage of luminal antigens. Environmental factors, such as intercurrent infections or the use of nonsteroidal anti-inflammatory drugs specifically acting at this level could represent the cause setting off the onset of IBD. The inflammatory response in IBD is

characterized by an increased production of proinflammatory cytokines (TNF-α, IL-6, IL-1β) supported by cytokines produced by T cells. In CD, IL-12/23 stimulates the production of IFN-γ, which plays a leading role in giving rise to the (Th1-mediated) inflammation. In UC, the increased production of IL-5 and IL-13 plays a leading pathogenetic role in the genesis and chronicization of the (Th2-like-mediated) inflammation (Fuss I.J. *et al.*, 1996, 2004; Bouma G. and Strober W., 2003).

A high expression of IL-17 and IL-23 has been found in CD and UC (Rovedatti L. *et al.*, 2009), even though it would seem that IL-23 regulates the Th effector subsets in a different way in CD and in UC, increasing the IFN-γ (Th1 cytokine) production by T CD4 cells + of the lamina propria in patients with CD and the production of IL-17 in UC (Kobayashi T. *et al.*, 2008). The chronicization of the inflammation is also supported by the presence of a relatively inadequate regulatory response. In fact, it has been shown that, in active IBDs, the effector cells have an increased SMAD7 (an intracellular inhibitor of TGF-β signaling) expression that is likely to prevent the TGF-β-mediated suppression effect of the regulatory T cells (Monteleone G. *et al.*, 2001; Boirivant M. *et al.*, 2006). Therefore, during the active phases of the disease, the regulatory response could prove ineffective in consequence of the presence of effector cells resistant to the inhibition of the regulatory cells. In short, results achieved up to now show that in IBD there is an increase in the inflammatory infiltration with an increase in the local production of proinflammatory cytokines with respect to the controls. Quite recently, an insufficient acute inflammatory response has been observed in patients with CD, leading to the development of chronic inflammation as a result of the partial inefficiency of the innate response (Smith A.M. *et al.*, 2009).

Environmental triggers

Studies have implicated several environmental triggers in the pathogenesis of IBD. These factors include smoking, which is protective in ulcerative colitis but detrimental in Crohn's disease, diet, the use of antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs), stress and infection. Unfortunately, the mechanisms by which these factors initiate the onset of disease or reactivate quiescent IBD are not well understood. These triggering factors alter mucosal barrier integrity, immune responses, or the luminal microenvironment, each of which have an impact on susceptibility to inflammation.

Infection and NSAIDs can transiently initiate an acute inflammation, break the mucosal barrier and activate innate immune responses.

Smoking is perhaps the most thoroughly documented environmental contributor to IBD, but its opposite effect on Crohn's disease and ulcerative colitis, respectively, is difficult to understand. Nicotine, carbon monoxide and hypoxia have all been suggested to be mediators of the effects of smoking on IBD (Birrenbach T. and Bocker U., 2004; Cosnes J., 2004).

2. AIM

In these studies, we meant to investigate the relationships between intestinal microbiota and immune mucosal response. In particular, we delved into the immune mucosal response to the increase in intestinal permeability, a condition that is normally considered to promote a local and systemic inflammatory response to an increased load of the bacterial components of the microbiota.

In fact, the increase in intestinal permeability is considered a condition that contributes to the pathogenesis of the inflammatory bowel diseases (ulcerative colitis and Crohn's disease), and the *NOD2* gene polymorphism, considered a risk factor for the onset of Crohn's disease, is associated with the presence of an increased intestinal permeability not only in patients, but also in relatives who are carrier of the same genetic polymorphism. Therefore, in these studies we have investigated the mucosal response mechanisms that follows a transitory increase in the permeability of the epithelial colon barrier. We performed our studies in control mice, and in mice with deletion of the *Nod2* gene.

3.RESULTS

A Transient Breach in the Epithelial Barrier Leads to Regulatory T Cell Generation and Resistance to Experimental Colitis

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Monica Boirivant[1], Antonello Amendola[1], Alessia Butera[1], Massimo Sanchez[2], Lili Xu[3] , Mariarosaria Marinaro[1], Atsushi Kitani[3], Claudia Di Giacinto[1] , Warren Strober[3], Ivan J. Fuss[3]

[1]Immune-Mediated Diseases Section, Department of Infectious, Parasitic, and Immune-Mediated Diseases, and

[2]Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy and

[3]Mucosal Immunity Section, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

Short title: Epithelial Barrier and Regulatory T Cells

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Abbreviations: lamina propria mononuclear cells (LPMC), Latency-Associated Peptide (LAP)

Correspondence: Monica Boirivant. Immune-Mediated Diseases Section, Department of Infectious, Parasitic, and Immune-Mediated Diseases. Istituto Superiore di Sanità, Viale R. Elena, 299. 00161 Roma, ITALY. Phone: 39-06-49902976, Fax: 39-06-49902931, e-mail: monica.boirivant @iss.it

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Abstract

Background and Aims: Previous studies have indicated that a defective epithelial barrier leads to inflammation of the underlying lamina propria. Nevertheless, it is likely that physiologic breaks in the barrier must occur for homeostatic regulatory T cells to develop. We determined the effect of agents that disrupt epithelial tight junctions (ethanol and AT1002, a *Vibrio cholerae* zonula occludens toxin hexapeptide) on regulatory T-cell induction and resistance to induction of colitis by trinitrobenzene sulfonic acid (TNBS). *Methods*: The effects of ethanol and AT1002 on colon immune function were evaluated by their capacity to induce direct phenotypic or functional changes in effector and regulatory cell populations and their indirect effect on the development of TNBS-induced colitis. The basis of regulatory cell development was evaluated with *in vitro* studies of isolated dendritic cell populations. The role of innate immunity was evaluated by *in vivo* gene silencing studies utilizing Toll-like receptor (TLR)-2-specific small interfering RNA (siRNA). *Results*: Both ethanol and AT1002 induced persistent latencyassociated peptide-positive $CD4^+$ regulatory T cells that, as shown in adoptive transfer studies, render mice resistant to the induction of TNBS colitis. The development of these cells requires the presence of an intact microflora and the activity of $CD11c^+$ dendritic cells. Their induction is also influenced by innate immune factors operating through TLR-2, because attenuation of TLR-2 signaling by *in vivo* TLR-2 siRNA administration prevents their development. *Conclusion*: A mild and/or transient breach in epithelial barrier function leads to dominant regulatory T-cell responses that protect the mucosa from inflammation.

Introduction

The commensal organisms of the gut microflora act via a number of mechanisms to reduce colonization of the mucosal surface by pathogenic organisms and the inflammation caused by these organisms.[1] However, if and when these organisms penetrate the epithelial cell layer they are themselves able to cause inflammation. This was shown over a decade ago with mice that exhibit inflammation in areas subjacent to epithelium with tight junction disruption due to expression of a dominant N-cadherin.[2] More recently, an inflammatory state with a similar pathogenesis was observed in mice with epithelial cell-specific NEMO deficiency and thus defective NF-κB function that manifest impaired epithelial barrier function due to epithelial cell apoptosis and ulcer formation.[3] These and similar findings lead to the view that an intact epithelium is a bulwark against the development of GI inflammation and that various types of epithelial cell defects that impairs barrier function is a potential cause of such inflammation.[4] It is important to note, however, that the normal epithelium does allow limited exposure of mucosal immune elements to antigens in the commensal microflora.[5] One possible reason for this apparent subversion of epithelial cell barrier function may be the need of the mucosal system to develop immunological tolerance toward antigens in the commensal microflora, since in the absence of such tolerance mucosal inflammation is likely to occur.

In the present study, we investigated this possibility by asking if a transient increase in mucosal permeability induced by either rectal administration of ethanol or a *Vibrio Cholerae* zonula occludens toxin hexapeptide (AT1002) (the latter a substance that specifically affects tight junction integrity) would have any effect on the subsequent ability to induce and sustain (TNBS)-induced colitis. We found that both types of treatment, while itself inducing a mild and transient inflammatory response, led to the induction of regulatory T cells and a state of resistance to the induction of TNBS-colitis. These findings thus provide formal proof that limited exposure of the mucosal immune system to the microflora is an important mechanism of tolerance induction.

Materials and Methods

Mice

Studies were performed in 5- to 6-week-old male SJL or C57BL10 mice obtained from Charles River (Calco, Italy), Harlan (S. Pietro al Natisone [UD], Italy), or Jackson Laboratories (Bar Harbor, ME) and maintained in the animal facilities at the Istituto Superiore di Sanità (Rome, Italy) or at the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). All animal studies were approved by the Animal Care and Use Committees of the Istituto Superiore di Sanità and the National Institutes of Health.

Study Design

Mice were administered either 50% ethanol or AT1002 (250 μg/mouse in 150 μL of phosphate-buffered saline) (the latter kindly provided by A. Fasano, University of Maryland School of Medicine, Baltimore, MD) via a 3.5F catheter inserted into the rectum as previously described.[6] Mouse weight changes and histologic appearance of the colonic tissue were then determined at various time points. In some experiments, mice were killed at appropriate time points to obtain lamina propria mononuclear cells (LPMCs) for various studies described in the text. For studies of effects of epithelial barrier disruption on colitis, mice administered 50% ethanol, AT1002, or no treatment were subsequently administered TNBS 2.5 mg delivered in 150 μL of 50% ethanol (Sigma-Aldrich, St. Louis, MO and Fluka, Milan, Italy) per rectum as previously described.[6] The occurrence and course of colitis in the different groups of animals were evaluated as described in the text.

Isolation and Culture of Lamina Propria Mononuclear Cells (LPMC)

LPMCs were isolated from freshly obtained colonic specimens using a modification of the method described by van der Heijden and Stok.[7] In some experiments, $CD11c^+$ and $CD4^+$ cells were purified by magnetic cell sorting using CD11c (N418) microbeads or a $CD4^+$ T-cell Isolation Kit (Miltenyi Biotec, Calderara di Reno, Italy or R&D Systems, Minneapolis, MN) following the manufacturer's instructions. The purity of LPMC $CD11c^+$ and $CD4^+$ cells was >90% and >95%,

respectively, as assessed by immunofluorescence staining. To measure the capacity of LPMCs to produce T-cell cytokines, the LPMCs were cultured at 1 x 10^6 cells/mL in 48-well plates (Costar; Corning Inc, Turin, Italy) coated or uncoated with murine anti-CD3e antibody (clone 145-2C11; BD-PharMingen, San Diego, CA) and containing complete media supplemented with 1 μg/mL of soluble anti-CD28 antibody (clone 37.51; PharMingen) as previously described.[6] To measure the capacity of LPMCs to produce interleukin (IL)-12p70, the LPMCs were cultured at 1 x 10^6 cells/mL in 48-well plates in media containing Staphylococcus aureus Cowan strain I (1:10,000; Calbiochem, Gibbstown, NJ), and interferon (IFN)-γ (1000 U; R&D Systems). After 48 hours of culture, supernatants were harvested and assayed for the presence of cytokines (IL-12p70, IFN- γ, IL-10) by enzyme-linked immunosorbent assays, conducted as previously described.[6]

In Vitro Stimulation of $CD4^+$ Lamina Propria T Cells by $CD11c^+$ Lamina Propria Dendritic Cells

LPMCs obtained from 50% ethanol-treated or untreated mice were subjected to magnetic bead sorting (Miltenyi Biotec) to obtain $CD11c^+$ and $CD4^+$ cells. A total of 2 x 10^5/mL of the $CD4^+$ cells from untreated mice was cocultured with 1 x 10^5/mL $CD11c^+$ cells from 50% ethanol–treated mice or from untreated mice in the presence or absence of 20 μg/mL of anti-IL-10R monoclonal antibody (1B1.3; DNAX Research, Palo Alto, CA). At the end of 48 hours of culture, the cells were collected, washed, and stained for latency-associated peptide (LAP) expression.

LPMC Immunofluorescence Staining

Freshly isolated and washed LPMCs were subjected to Fc block with anti-CD16/CD32 (BD PharMingen) and then stained with biotinylated anti-LAP (affinity-purified biotinylated goat anti-LAP polyclonal antibody; R&D Systems) or biotinylated normal goat immunoglobulin G (R&D Systems) as previously described.[6] In some experiments, intracellular Foxp3 expression was evaluated using the APC anti-mouse/rat Foxp3 Staining Set (eBioscience, San Diego, CA) following the manufacturer's protocol.

Intestinal Permeability Studies

To measure colonic permeability, mice were administered 50% ethanol per rectum. At daily time points, mice then received per rectum 2 mg/10 g body wt fluorescein isothiocyanate-conjugated dextran dissolved in purified water (4000 mol wt; Sigma-Aldrich). Whole blood was obtained via eye bleed 1 hour after fluorescein isothiocyanate dextran administration. Blood samples were heparinized and centrifuged at 3000 rpm to obtain sera; the latter was then analyzed by fluorometry using a Victor^3 (485/535 nm, gain 1480) plate reader (Perkin-Elmer, Waltham, MA).

Depletion of Gut Commensal Microflora

Depletion of gut microflora was accomplished as previously described by the administration of ampicillin (1 g/L; Pharmacia, Milan, Italy), vancomycin (500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L) in drinking water for 4 weeks.[8] Evaluation of the effect of antibiotic treatment on intestinal microflora was per formed with standard microbiological methods by Plaisant srl (Rome, Italy). Briefly, fecal matter was removed from colons using sterile technique, placed in 15 mL conical tubes with thioglycolate, weighed, and vortexed until homogeneous. Contents were diluted and plated on universal and differential media for the growth of anaerobes and aerobes. Colonies were counted after incubation at 37 °C for 48 hours (aerobes) and 72 hours (anaerobes). After counting, colonies were picked and identified by biochemical analysis, morphologic appearance, and Gram staining.

Adoptive Transfer of LPMC or Lamina Propria CD4$^+$ Cells Depleted of LAP-Positive T Cells

LPMCs or lamina propria (LP) CD4$^+$ T cells isolated from the colons of mice treated with 50% ethanol were depleted of LAP-positive cells by using a CELLection Biotin Binder Kit (Dynal Biotech ASA, Invitrogen SRL, San Giuliano Milanese, Italy). A total of 3.0 x 10^5 LPMCs, LP CD4$^+$ T cells, LAP cell-depleted LPMCs, or LAP-depleted LP CD4$^+$ T cells were injected via tail vein as described.[6]

Histological Assessment of Colitis

Tissues removed from mice at indicated time points were fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) and then embedded in paraffin, cut into tissue sections, and stained with H&E. Stained sections were examined for evidence of colitis using criteria described in detail previously.[6]

In Vivo Inhibition of Toll-like Receptor 2 by Administration of Toll-like Receptor 2-specific Small Interfering RNA

Sequences of toll-like receptor (TLR)-2–specific small interfering RNA (siRNA) for use in TLR2 silencing studies (Dharmacon, Lafayette, CO) were as follows: 5'-UCAAUGGGCUCGGCGAUUUUU-3' and 5'-AAGAUGCGCUUCCUGAAUUUU-3'. The sequence for the control siRNA was as follows: 5'-UAAGGCUAUGAAGAGAUACUU- 3'. For *in vivo* transfection, 1 mg of siRNA was encapsulated in HVJ-E vector as previously described.[9] Mice were administered siRNA according to protocols described in the text.

Western Blot

Western blot was performed with the lysates of adherent splenocytes using rabbit anti-mouse TLR2 or TLR4 antibodies (eBioscience) and horseradish peroxidise-conjugated anti-rabbit immunoglobulin G antibody.

Statistical Analysis

Assessment of statistical differences was determined by parametric Student t test and nonparametric Mann-Whitney test as appropriate.

Results

Intrarectal Ethanol Administration Leads to Mild Colonic Inflammation and Increased Colonic Permeability

Whereas induction of hapten-induced colitis requires administration of the hapten in an ethanol carrier to break the epithelial barrier, we reasoned that administration of ethanol in the absence of the effector cell stimulant (the hapten) may have a contrary effect: the induction of regulatory cells. In initial studies to investigate this possibility, we determined the effect of intrarectal administration of 50% ethanol in distilled water (50% ethanol) (150 μL), the same dose of ethanol normally used to induce TNBS colitis. As shown in Figure 1A and B, such ethanol administration resulted in a small but transient loss of weight compared with (untreated) control mice. This was accompanied at day 3 by the transient appearance of a mild inflammatory infiltrate in the colonic LP, as well as scattered (mild) epithelial cell disruption that resolved by day 7. In addition, as shown in Figure 1C, the infiltrating LPMCs were capable of producing significantly increased levels of IFN-γ and IL-10, but not IL-4 (the latter data not shown), as compared with cells extracted from control mice. Of interest, substantial amounts of IL-10 were secreted by unstimulated cells, suggesting that such IL-10 was being produced by cells activated *in vivo*. Finally, as shown in Figure 1D, this inflammatory response to intrarectal ethanol administration was accompanied by a transient change in colonic permeability, as measured by dextran/fluorescein isothiocyanate uptake into the circulation. In related studies, we also determined the effect of a second rectal administration of 50% ethanol 12 days after the initial administration. As shown in Supplementary Figure 1A and B (see Supplementary material online at www.gastrojournal.org), the second administration induced effects similar to those observed after the first administration, although in this case the inflammation induced was somewhat diminished.

Intrarectal Ethanol Administration Is Accompanied by the Induction of LAP-Positive $CD4^+$ T Cells

In further studies, we determined the percentage of cells in the LP bearing markers associated with regulatory T cells. As shown in Figure 2, 3 days after intrarectal ethanol administration, there was a significant increase in the proportion of both $CD4^+CD25^+$ T cells and $CD4^+$ T cells bearing latent

transforming growth factor (TGF)-β (LAP) on their cell surface ($CD4^+$ LAP-positive T cells) compared with LP cells from control mice not treated with ethanol. These cell populations were partially overlapping in that only about 25% of the LAP-positive cells were also $CD25^+$ in LPMCs obtained from both mice treated and not treated with ethanol (data not shown). Cells with a similar array of markers were seen after a second administration of ethanol (Supplementary Figure 1C; see Supplementary material online at www.gastrojournal.org).

Because Foxp3 is a transcription factor associated with a major type of regulatory T cell, we also determined Foxp3 expression in the $CD4^+CD25^+$ and $CD4^+$ LAP positive T-cell populations appearing in the LPMCs after ethanol administration. We found that Foxp3 was coexpressed in 76% and 78% of $CD4^+CD25^+$ LP T cells in the LPMCs of untreated mice and ethanol-treated mice, respectively. In contrast, as shown in a representative study depicted in Supplementary Figure 2 (see Supplementary material online at www.gastrojournal.org), Foxp3 was coexpressed in only 7%-13% and 9%-18% of $CD4^+$ LAP-positive LP T cells from untreated and ethanol-treated mice, respectively.

To further characterize the ethanol-induced LAPpositive T-cell population, we performed marker studies on LPMCs obtained 3 days after ethanol administration, before and after *in vitro* depletion of LAP-positive T cells. We found that LPMCs after depletion exhibited a 50% reduction in the number of cells expressing LAP, but no change in the number of cells expressing Foxp3, and thus obtained further evidence that the LAP-positive T cells were largely Foxp3 negative (data not shown). In addition, the LPMCs after depletion exhibited an approximately 75% reduction in ex vivo production of IL-10 or TGF-β as compared with LPMCs before depletion; this suggested that LAP-positive T cells were also producers of these cytokines (data not shown).

Ethanol Administration Renders Mice Resistant to the Subsequent Induction of TNBS-Induced Colitis via the Induction of LAP-Positive $CD4^+$ T Cells

In further studies, we tested whether the regulatory T cells appearing after ethanol administration influence the severity of subsequently induced TNBS colitis. Accordingly, we compared the severity of colitis in mice with TNBS-induced colitis that were pretreated with intrarectal 50%

ethanol 12 days before with the severity of colitis in mice with TNBS-induced colitis not so pretreated. As shown in Figure 3A and B, mice pretreated with ethanol exhibited less severe TNBS-induced colitis, as indicated by their significantly lower loss of body weight, decreased histologic evidence of intestinal inflammation, and lower colitis scores, compared with mice not pretreated with ethanol. In addition, as shown in Figure 3C, cytokine production by LPMCs obtained from mice pretreated with ethanol exhibited a significant reduction in *in vitro* IFN-γ secretion compared with mice not pretreated with ethanol. Finally, observation of mice protected from induction of colitis for 7–10 days following resolution of colitis disclosed that pretreatment with ethanol did not lead to delayed induction of colitis.

In additional studies shown in Supplementary Figure 3 (see Supplementary material online at www.gastrojournal.org), we found that (1) the protective effect of ethanol administration was quite prolonged in that at least partial protection was still noted 5 weeks after such administration; (2) as shown in Supplementary Figure 4 (see Supplementary material online at www.gastrojournal.org), protection was not strain specific because it was also seen in C57BL/10 mice; and (3) as shown in Supplementary Figure 5 (see Supplementary material online at www.gastrojournal.org), correlating with the fact that ethanol administration is associated with increased ethanol administration in IL-10-deficient C57BL/10 mice did not lead to protection from subsequent induction of TNBS colitis.

To determine if the previously described ethanol-induced resistance to TNBS-induced colitis was indeed due to regulatory T cells, we next performed cell transfer studies in which naive mice were adoptively transferred LPMCs or purified LP $CD4^+$ T-cell populations from ethanol-treated mice before induction of TNBS colitis. In these studies, the transferred cells were 65%-80% depleted of LAP-positive cells as determined by flow cytometric studies. As shown in Figure 4A and B and Supplementary Figure 6 (see Supplementary material online at www.gastrojournal.org), naive SJL mice administered LPMCs or purified LP $CD4^+$ T cells obtained from ethanol-treated mice and then depleted *in vitro* of LAP-positive cells exhibited a similar severity of TNBS-induced colitis as control mice administered LPMCs from mice with TNBS-induced colitis or mice with TNBS-induced colitis who had not been subjected to ethanol pretreatment or cell transfer. In contrast, mice administered LPMCs or purified LP $CD4^+$ T cells from ethanol-treated mice not depleted of LAP-positive cells exhibited a reduced severity of colitis. Finally, as shown in Figure 4C, the effect of transfer of LPMCs or $CD4^+$ T cells or these cells depleted of LAP-positive cells from ethanol-treated mice correlated with *in vitro* LPMCs or $CD4^+$ T-cell production of both IL-12p70 and IFN-γ. It should be noted, however, that despite the use of very large numbers of mice, the number of data points obtained in these cytokine studies was limiting and could not be subjected to statistical

analysis; thus, these data are supportive of the statistically validated weight and colitis score data but are not in themselves definitive.

Taken together, these studies provide strong evidence that intrarectal ethanol administration induces LAP-positive $CD4^+$ regulatory T cells that then render mice resistant to the induction of TNBS colitis.

Induction of Transient Tight Junction Porosity in the Absence of Epithelial Damage by AT1002 Also Confers Resistance to Induction of TNBS Colitis

Intrarectal administration of 50% ethanol caused only minimal morphologic changes in the epithelial layer, so its ability to alter gut permeability was presumed to be due mostly to its previously observed effect on the integrity of tight junctions.[10] To obtain independent evidence that loss of tight junction integrity was sufficient to lead to expansion of regulatory cells and resistance to TNBS-induced colitis, we determined if pretreatment with an agent with a focused effect on tight junction integrity, the hexapeptide active domain of the *V cholerae* zonula occludens toxin AT 1002, caused the same effects as pretreatment with ethanol.[11,12] Accordingly, we subjected mice to intrarectal administration of AT1002 (250 μg in 150 μL phosphate-buffered saline) based on a previously established protocol for inducing increased intestinal permeability but no epithelial cell damage with this agent.[11,12] We found that intrarectal administration of AT1002 did not result in a loss of body weight (data not shown) and, likewise, as shown in Figure 5A, was accompanied by a mild LP inflammation without morphologic evidence of epithelial cell damage. However, as shown in Figure 5B and C, LPMCs obtained from mice 3 days after administration of AT 1002 did exhibit a mild increase in *in vitro* IFN-γ and IL-10 production as well as a significant increase in the percentage of $CD4^+$ LAP-positive T cells in the LPMC population. Finally, as shown in Figure 5D and E, mice pretreated with AT1002 and then administered TNBS 12 days later to induce TNBS colitis exhibited less severe TNBS colitis than untreated mice. These data show that changes in epithelial barrier function specifically due to a transient change in the integrity of the epithelial tight junctions are sufficient to cause the generation of LP LAP-positive T cells and the associated increased resistance to the development of TNBS colitis.

$CD4^+$ LAP-Positive Cells Expansion Is Dependent on the Presence of an Intact Bacterial Flora

To evaluate the influence of bacterial flora on ethanol-induced increases in LP LAP^+ cells, we treated a group of mice for 4 weeks with a cocktail of 4 antibiotics (see Materials and Methods) to partially sterilize the gastrointestinal tract before the administration of 50% ethanol. Such treatment led to an approximately 100-fold decrease (as measured by colonyforming units in samples of material from various parts of the colon) in both aerobic and facultative anaerobic bacteria; in addition, as assessed by standard bacteriologic studies, the residual flora was not qualitatively changed. As shown in Figure 6A, antibiotictreated mice lost significant weight following ethanol administration, and unlike mice not given antibiotics, they did not return to normal weight after 7 days. In addition, as shown in Figure 6B, the extracted LP population of antibiotic-treated mice displayed no increase in LAP-positive cells and, as shown in Figure 6C, transfer of LPMCs from these mice to recipients did not confer protection from TNBS-induced colitis. Thus, intrarectal administration of 50% ethanol requires the presence of an intact microflora for the induction of LAP-positive cells.

Myeloid Dendritic Cells Induce the Expansion of $CD4^+$ LAP-Positive T Cells

In further studies to determine the role of myeloid dendritic cells (DCs) in the protective effect induced by ethanol, we purified $CD11c^+$ DCs from LPMCs isolated from ethanol-treated and untreated mice and then cultured the DCs thus obtained with purified $CD4^+$ T cells from LPMCs isolated from naive untreated mice. Then, at the end of the culture period, we determined the number of LAP-positive cells by flow cytometry. As shown in Figure 7, in 2 independent studies, coculture of $CD11c^+$ DCs isolated from ethanol-treated mice with naive $CD4^+$ T cells, with no other stimulation *in vitro* provided, was associated with a higher percentage of LAP-positive cells as compared with the percentage of LAP-positive cells from coculture of $CD11c^+$ DCs isolated from untreated mice with naive $CD4^+$ T cells. Of interest, this $CD11c^+$ DC effect was dependent on the availability of IL-10 because addition of anti-IL-10R antibody prevented the increase in the number of LAP-positive cells (data not shown). These data suggest that myeloid DCs are responsible for the induction of $CD4^+$ LAP-positive cells.

TLR2 Signaling Is Critical for the Generation of LAP-Positive Regulatory Cells

Prior studies have established that TLR signalling is necessary for induction of regulatory T cells in mucosal tissues.[13,14] To examine the role of one such TLR, TLR2, in ethanol-induced induction of regulatory T cells, we determined the ability of mice with reduced TLR2 expression due to *in vivo* administration of TLR2-specific siRNA to develop regulatory T cells upon intrarectal ethanol administration. Accordingly, mice were pretreated with 100 μg of either TLR2-specific or control siRNA on the day before and on the day of intrarectal ethanol administration. As shown in Figure 8A, by Western blot analysis of spleen whole cell lysates, mice administered TLR2-specific siRNA exhibited decreased TLR2 but not TLR4 expression. As shown in Figure 8B, mice pretreated with TLR2-specific siRNA, but not control siRNA, exhibited a more protracted loss of weight upon intrarectal ethanol administration compared with mice treated with ethanol alone. In addition, as shown in Figure 8C, mice pretreated with TLR2-specific siRNA and killed at day 4 after ethanol administration did not exhibit a significant increase in $CD4^+$ LAP-positive LP T cells, whereas mice pretreated with control siRNA exhibited a significant increase. This correlated with the fact that, as shown in Figure 8D, LPMCs from mice administered TLR2-specific siRNA exhibited reduced IL-10 but no change in IFN-γ production upon stimulation with anti-CD3/anti-CD28 *in vitro* as compared with mice administered control siRNA. Finally, as shown in Figure 8E and F, mice administered TLR2-specific siRNA in association with ethanol did not exhibit resistance to induction of TNBS colitis, whereas mice administered control siRNA in association with ethanol did exhibit such resistance. These data thus indicate that the ethanol-induced generation of LAP-positive regulatory T cells is dependent on TLR2 signaling.

Discussion

In light of previous data showing that disruption of the epithelial barrier leads to inflammation of the LP,[2] it was quite unexpected that administration of 2 agents (ethanol and AT1002) that cause transient and mild disruption of barrier function is followed by the expansion of a persistent population of regulatory T cells that greatly increases resistance to the subsequent induction of TNBS colitis. The basis of such regulatory T-cell expansion became apparent in studies showing that such expansion depended on the presence of an unperturbed gut microflora and, in addition, was associated with the presence of LP DCs with a propensity to induce regulatory cells upon exposure to microflora antigens. It can therefore be concluded that transient and limited disruption in barrier function leads to a dominant regulatory immune response to gut microflora antigens and that the cross talk between the microflora and the mucosal immune system, even when somewhat exaggerated by epithelial barrier disruption, has a net anti-inflammatory effect.

The nature of the regulatory T cell induced by barrier disruption was also somewhat unexpected. The identifying feature of this cell is its expression of surface TGF-β in the form of TGF-β bound to LAP. Of note, while a substantial number of the LAP-positive regulatory T cells were $CD25^+$, a substantial number did not bear this marker. Perhaps more importantly, while most of the $CD25^+$ cells were Foxp3 positive, most of the LAP-positive cells were Foxp3 negative. This leads to the possibility that most of the LAP-positive regulatory cells developing under the circumstances studied here were actually more closely related to the Tr-1 regulatory cell than to the "natural" ($Foxp3^+$) regulatory cell, even though the latter cell has also been shown to bear surface LAP.[15] This view is favored by previous studies of regulatory LAP-positive cells developing in mice treated with probiotics showing that the regulatory cell involved was dependent on IL-10.[6] In addition, it is favored by our finding that IL-10–deficient mice do not display resistance to induction of TNBS colitis upon intrarectal preadministration of ethanol and induction of LAP-positive regulatory T cells by myeloid DCs *in vitro* is inhibited by anti-IL-10R. Finally, the existence of Tr-1-like regulatory cells bearing surface LAP is supported by recent studies showing that *in vitro* suppression by such cells is inhibited by anti-TGF-β as well as by studies showing that populations of LAP-positive cells lacking Foxp3 have suppressor function.[16,17]

Extensive *in vivo* depletion studies showing that plasmacytoid DCs were not necessary for ethanol-induced regulatory T cells (data not shown) led us to the supposition that myeloid DCs were critically necessary for such induction. This supposition was subsequently supported by *in vitro* studies showing that ethanol administration led to the appearance of myeloid DCs with the capacity

to induce $CD4^+$ LAP-positive T cells. Previous studies of mucosal myeloid DC populations showing that mucosal DCs that produce TGF-β or that bear surface LAP support the development of regulatory T cells in the mucosa are in accord with this finding, although in these cases the regulatory cell induced was a $Foxp3^+$ cell.[18] Thus, in this case, the capacity of the inducing DC to produce IL-10 rather than TGF-β may be its most salient feature. This is supported by the fact that, as already mentioned, *in vitro* induction of such cells by myeloid DCs was blocked by the addition of anti-IL-10R to the culture. In addition, the finding that ethanol-induced regulatory cells do not develop in the absence of effective TLR2 signaling, a potent inducer of IL-10, also supports the importance of the latter cytokine in regulatory T-cell induction.

In summary, the present studies establish that increased exposure to intestinal microflora or their products via a transiently disrupted epithelial barrier can have the paradoxical effect of increasing the resistance of the mucosa to inflammation via the induction of regulatory T cells. Such cells then have the capacity to protect mice against the development of TNBS-induced colitis, a mucosal inflammation depending on both innate and adaptive immune responses. This finding is important to our understanding of the origin of human inflammatory bowel disease because it is assumed by some investigators that subtle changes in barrier function alone may be a cause of such disease. On the contrary, these findings suggest that in some cases subtle changes may have a protective effect.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of G*astroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.07.028.

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Figure Legends

Figure1. Effects of intrarectal administration of 50% ethanol (150 μL). (A) Weight curves of ethanol-treated and untreated mice. Each point represents the cumulative mean weight ± SE from 3 independent experiments, each consisting of groups of 10 mice. *P<.05, ethanol-treated mice versus untreated mice. (B) Representative H&E-stained microscopic sections (original magnification x50) of colon at different time points after ethanol administration: (a) ethanol-treated mouse at day 1, (b) day 3, (c) day 5, and (d) day 7after intrarectal ethanol administration. (*Inset*) Microscopic view of epithelium (x400). (C) *In vitro* IFN-γ and IL-10 production by LPMCs isolated from the colons of SJL mice 3 days after ethanol administration. Cultures were performed in the absence of any stimulant (*open bars*) or in the presence of anti-CD3/CD28 (*closed bars*). Data represent the mean ± SE from 3 independent experiments. In each experiment, cultures of pooled cells extracted from 5 mice per group were performed. IFN-γ, P = .02 anti-CD3/28 stimulated cells from 50% ethanol–treated versus untreated mice; IL-10, P = .03 unstimulated cells from 50% ethanol–treated versus untreated mice, P = .007 anti-CD3/28 stimulated cells from 50% ethanol–treated versus untreated mice. (D) Intestinal permeability as measured by rectal administration of fluorescein isothiocyanate/dextran. *P < .05 versus day 0.

Figure 2. Effect of a single intrarectal administration of 50% ethanol on the generation of $CD3^+CD4^+$ LP T cells expressing CD25 and LAP. Mice were killed at day 3 after treatment. Data indicate the percentage of positive cells in the $CD3^+CD4^+$ gated population. The percentage of CD4-positive cells was not statistically different in the 2 groups (ethanol-treated mice, 32.9% ± 3.18%; untreated mice, 34.16% ± 4.8%).

Figure 3. Effect of intrarectal administration of 50% ethanol on induction of TNBS colitis. (A) Weight changes of mouse groups after TNBS administration. Each point represents the cumulative mean weight (±SE) from 5 separate experiments. In each experiment, each group consisted of at least 5 mice. *P< .05, 50% ethanol-pretreated mice versus untreated mice. (B) Histologic evaluation of the effect of preadministration of 50% ethanol on subsequent TNBS-induced colitis. Mice were killed at 3 days after TNBS administration. (a) Photomicrograph (original magnification x50) of an H&Estained paraffin section of a representative colon from a mouse after induction of TNBS colitis. (b) Photomicrograph (original magnification x50) of an H&Estained paraffin section of a representative colon from a mouse after induction of TNBS colitis following preadministration of 50% ethanol. Histologic scores (see scoring criteria in Materials and Methods) of colons of the

various groups of mice after induction of TNBS colitis; each group consisted of 5 mice. (C) *In vitro* IFN-γ production by LPMCs isolated from the colons of mice on day 3 after TNBS administration. Cultures were performed in the absence of any stimulant (*open bars*) or in the presence of anti-CD3/CD28 (*closed bars*). Data represent the mean ± SE from 4 independent experiments. In each experiment, cultures of pooled cells extracted from 5 mice per group were performed. IFN-γ, P = .03 anti-CD3/28 stimulated cells from 50% ethanol-pretreated versus untreated mice.

Figure 4. Depletion of LAP-positive cells from donor LPMCs or purified LP $CD4^+$ T cells of ethanol-pretreated mice before adoptive cell transfer abolishes the protective effect of the LPMCs. (A) Weight changes of groups of mouse recipients of LPMCs obtained from mice treated with ethanol (*diamonds*), recipients of LAP-positive cell-depleted LPMCs obtained from mice treated with ethanol (*squares*), recipients of LPMCs obtained from mice treated with TNBS (*triangles*), and mice treated with TNBS alone (*x*). Recipient mice were administered TNBS per rectum 5 days after adoptive transfer of LPMCs. Each experimental group consisted of at least 5 mice, and each data point represents the mean value ± SE for each group. Histologic scores of colons of mice in the various groups were obtained as described in Materials and Methods. *P <.05 mice recipients of Eth LPMC vs mouse recipients of Eth LPMC LAP-, TNBS LPMC, and mice treated with TNBS alone. (B) Weight changes of groups of mouse recipients of LPMCs obtained from mice treated with ethanol (*diamonds*), recipients of purified LP $CD4^+$ T cells obtained from mice treated with ethanol (*closed circles*), recipients of LAP^+ cell-depleted LP $CD4^+$ T cells obtained from mice treated with ethanol (*open circles*), and mice treated with TNBS alone (*x*). Recipient mice were administered TNBS per rectum 5 days after adoptive transfer of LPMCs. Each experimental group consisted of at least 4 mice, and each data point represents the mean value ± SE for each group. Histologic scores were obtained as described in Materials and Methods. P < .05 mice recipients of Eth LPMC and Eth $CD4^+$ vs mouse recipients of Eth $CD4^+$, LAP-, and mice treated with TNBS alone. (C) Cytokine production by LPMCs isolated from recipient mouse colons 4 days after induction of TNBS colitis. Cultures were performed in the absence of any stimulant (*open bars*) or in the presence of IFN-γ–positive SAC or anti-CD3/CD28 for IL-12 and IFN-γ stimulation (*closed bars*), respectively. Data shown represent values obtained from a representative experiment of an LP $CD4^+$ T-cell transfer study and 2 experiments from an LPMC transfer study. In each experiment, culture supernatants were obtained from pooled cells extracted from all mice per group.

Figure 5. Effect of intrarectal administration of AT1002. (A) Photomicrographs (original magnification x100) of H&E-stained paraffin sections of (a) an untreated colon and (b) a representative colon 3 days after intrarectal administration of AT1002. (B) *In vitro* IFN-γ and IL-10 production by LPMCs isolated from the colons of mice on day 3 after AT1002 administration. Cultures were performed in the absence of any stimulant (*open bars*) or in the presence of anti-CD3/CD28 (*closed bars*). Data represent the mean ± SE from 3 separate experiments. In each experiment, cultures of pooled cells extracted from 5 mice per group were performed. (C) Cells expressing CD25 and LAP in LPMCs isolated 3 days after AT1002 administration (*closed bars*) or untreated mice (*open bars*). Data indicate the percentage of positive cells in the $CD3^+CD4^+$ gated population. Data represent the mean ± SE from 3 separate experiments. P = .02, LAP-positive cells of AT1002-treated versus untreated mice. (D) Weight changes of mice with TNBS-induced colitis after intrarectal administration of AT1002. Each point represents the cumulative mean ± SE from 3 separate experiments. In each experiment, each group consisted of at least 5 mice. *P < .05, AT1002-pretreated mice versus untreated mice. (E) Histologic scores of colons of groups of mice killed at day 3 after TNBS administration; see scoring criteria in Materials and Methods.

Figure 6. Effect of depletion of commensal microflora. (A) Weight changes after 50% ethanol administration. Each point represents the cumulative mean weights ± SE from 2 different experiments; in each experiment, each group consisted of at least 5 mice. *P = .04, 50% ethanol versus antibiotics/50% ethanol. (B) CD25 and LAP expression in LPMCs 3 days after intrarectal ethanol administration from mice that were administered antibiotics (*open bars*) or untreated mice (*closed bars*). LAP expression in LPMCs of untreated naive mice (*grey bars*). Data indicate the mean percentage of positive cells in the $CD3^+CD4^+$ gated population. (C) Weight changes of groups of mice recipients of LPMCs obtained from mice treated with ethanol (*open triangles*), recipients of LPMCs obtained from mice treated with antibiotics/ethanol (*closed triangles*), and mice treated with TNBS alone (*x*). Recipient mice were administered TNBS per rectum 5 days after adoptive transfer of LPMCs. Each experimental group consisted of at least 5 mice, and each data point represents the mean value ± SE for each group.

Figure 7. Percentages of LAP-positive cells in purified LP $CD4^+$ T-cell populations following coculture for 48 hours with $CD11c^+$ dendritic cells isolated from the LP of mice administered 50% ethanol or untreated mice.

Figure 8. Effect of administration of siRNA specific for TLR2 on ethanol induction of LAP-positive cells. (A) Western blot analysis of TLR2 and TLR4 expression in untreated mice (*lane 1*), mice administered 50% ethanol (*lane 2*), mice administered 50% ethanol that received concomitant control siRNA (*lane 3*), or mice administered 50% ethanol that received concomitant siRNA for TLR2 (*lane 4*). (B) Weight changes of groups of mice after administration of siRNA to TLR2 or control siRNA to mice pretreated with 50% ethanol. Each point represents the cumulative mean weight ± SE from groups consisting of at least 5 mice. *P < .05, siRNA TLR2/50% ethanol versus 50% ethanol. (C) LAP expression in LPMCs isolated 4 days after intrarectal ethanol administration of mice administered concomitant TLR2 siRNA or control siRNA. Data indicate the mean ± SE percentage of positive cells in the $CD3^+CD4^+$ gated population. (D) *In vitro* IFN-γ and IL-10 production by LPMCs isolated from the colons of mice 4 days after ethanol concomitant siRNA administration and cultured in the presence of anti-CD3/CD28. Data represent the mean ± SE from 2 separate experiments comprising data from cultures of pooled cells extracted from 5 mice per group. (E) Weight changes of groups of mice after TNBS administration. Each point represents the cumulative mean weight ± SE from 2 separate experiments in which each group consisted of at least 5 mice. *P < .05, siRNA TLR2/50% ethanol/TNBS versus 50% ethanol/TNBS. (F) Photomicrograph (original magnification x50) of an H&E-stained paraffin section of a representative colon from each group after induction of TNBS colitis.

Supplementary Figure Legends

Supplementary Figure 1. Effect of a second intrarectal administration of 50% ethanol on regulatory T-cell development. To determine the effects of a second intrarectal administration of 50% ethanol, mice were administered 50% ethanol 12 days after the first administration. (A) Weight changes of untreated mice (*open bars*) and mice administered ethanol a second time (*closed bars*). Each point represents the cumulative mean weight ± SE from 5 separate experiments in which each group contained at least 5 mice; *P < .05 for mice that received a second 50% ethanol administration versus untreated mice. The mice receiving a second administration of ethanol exhibited weight loss compared with untreated mice; however, in this case, they returned more quickly to their original weight than after a single administration of ethanol. (B) *In vitro* IFN-γ and IL-10 production by LPMCs isolated from the colons of mice that received a second administration of ethanol versus untreated mice. Data represent means from 4 separate experiments; in each experiment, cultures consisted of pooled cells extracted from 5 mice per group. LPMCs extracted from mice 3 days after a second administration of 50% ethanol (*closed bars*) exhibited increased IFN-γ and IL-10 production, but these increases were not as great as after an initial administration of ethanol. (C)

Percentage of $CD4^+CD25^+$ T cells and $CD4^+$ LAP-positive T cells in the $CD3^+CD4^+$ gated LPMC population. $CD4^+CD25^+$ T cells and $CD4^+$ LAP-positive T cells were increased compared with these cells in LPMCs from untreated mice, but there was no difference between these values and those obtained after a single administration of ethanol.

Supplementary Figure 2. Phenotypic characterization of LAP and Foxp3 in $CD3^+CD4^+$ gated T cells of untreated or ethanol-treated mice. LP cells isolated from untreated and ethanol-treated mice were stained with LAP and Foxp3-specific antibodies and analyzed by fluorescence-activated cell sorting.

Supplementary Figure 3. Duration of protective effect of ethanol administration. Different groups of mice were administered TNBS intrarectally on day 12, 24, and 36 after intrarectal administration of 50% ethanol (*closed circles*) or phosphate-buffered (*open circles*). Groups of mice consisted of at least 5 mice.

Supplementary Figure 4. Effects of intrarectal administration of 50% ethanol are not strain specific. (A) Weight curves of ethanol-treated and untreated C57BL10 mice. Each point represents the cumulative mean weight ± SE, each consisting of groups of 10 mice. *P < .05, ethanol-treated mice versus untreated mice. (B) Effect of a single intrarectal administration of 50% ethanol on the generation of $CD3^+CD4^+$ LP T cells expressing LAP. Mice were killed at day 3 after treatment. Data indicate the percentage of positive cells in the $CD3^+CD4^+$ gated population. (C) Effect of intrarectal administration of 50% ethanol on induction of TNBS colitis. Weight changes of mouse groups after TNBS administration. Each point represents the cumulative mean weight (±SE). In each experiment, each group consisted of at least 5 mice. *P < .05, 50% ethanol-pretreated mice versus untreated mice.

Supplementary Figure 5. The effect of administration of 50% ethanol on resistance to TNBS-induced colitis is IL-10 dependent. Weight curves of ethanol-treated and untreated wild-type (WT) C57BL10 or C57BL10 IL-10 KO mice after TNBS administration. Each point represents the cumulative mean weight (±SE) of at least 5 mice per group. Mice were killed at 3 days after TNBS administration.

Supplementary Figure 6. Depletion of LAP-positive cells from donor LPMCs or purified LP $CD4^+$ T cells of ethanol-pretreated mice before adoptive cell transfer abolishes the protective effect. (A) Histology of (a) groups of mouse recipients of LPMCs obtained from mice treated with ethanol, (b) recipients of LAP-positive cell-depleted LPMCs obtained from mice treated with ethanol, (c) recipients of LPMCs obtained from mice treated with TNBS, and (d) mice treated with TNBS alone. Recipient mice were administered TNBS per rectum 5 days after adoptive transfer of LPMCs. Recipients of LPMC cells from LAP-depleted or TNBS-induced colitis mice did not differ in severity compared with mice treated with TNBS alone; recipients of LPMCs from ethanol-treated mice revealed only minimal inflammatory changes. (B) Histology of (a) groups of mouse recipients of LPMCs obtained from mice treated with ethanol, (b) recipients of purified LP $CD4^+$ T cells obtained from mice treated with ethanol, (c) recipients of LAP-positive cell-depleted LP $CD4^+$ T cells obtained from mice treated with ethanol, and (d) mice treated with TNBS alone. Recipient mice were administered TNBS per rectum 5 days after adoptive transfer of LPMCs. Recipients of LP $CD4^+$ T cells from LAP-depleted ethanol-treated mice did not differ in severity compared with mice treated with TNBS alone; recipients of LPMCs or purified LP $CD4^+$ T cells from ethanol-treated mice revealed only minimal inflammatory changes.

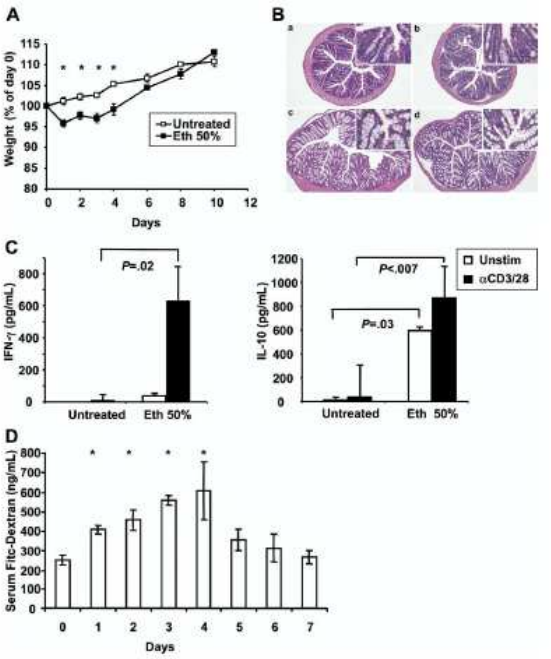
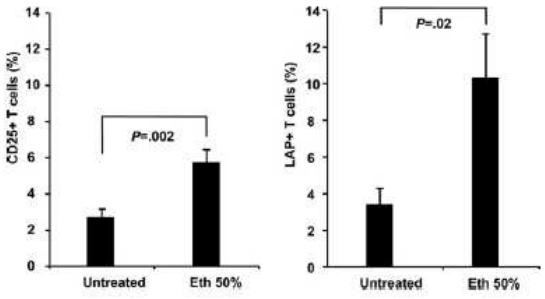


Figure 1



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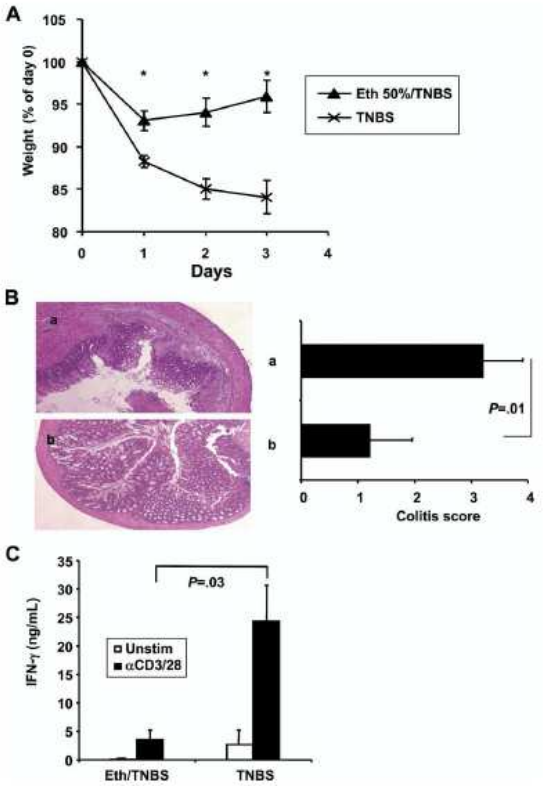

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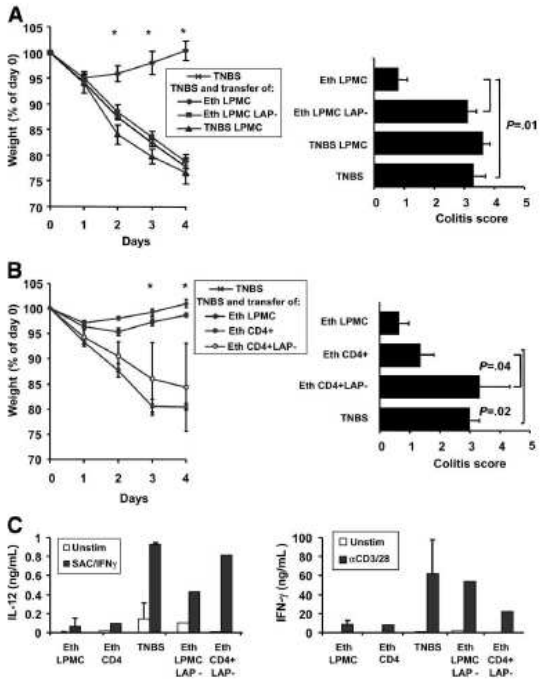

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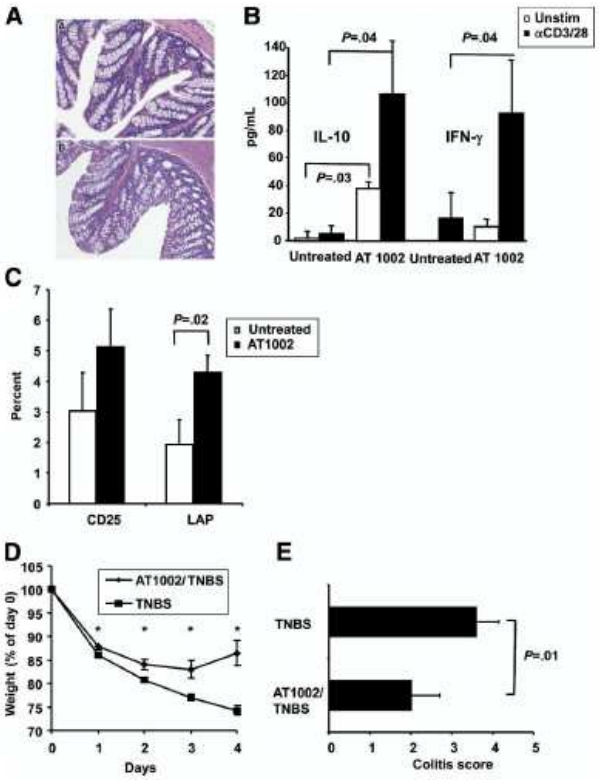

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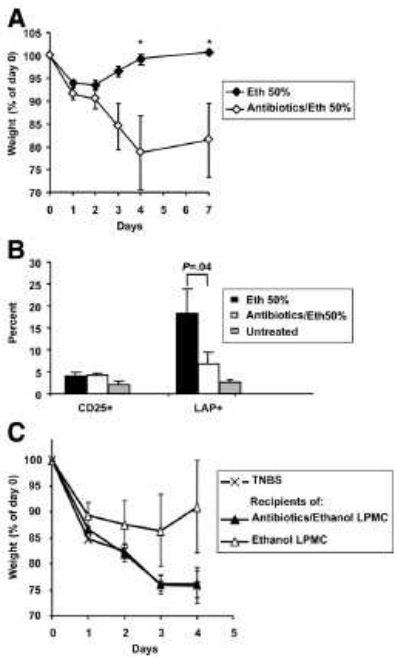

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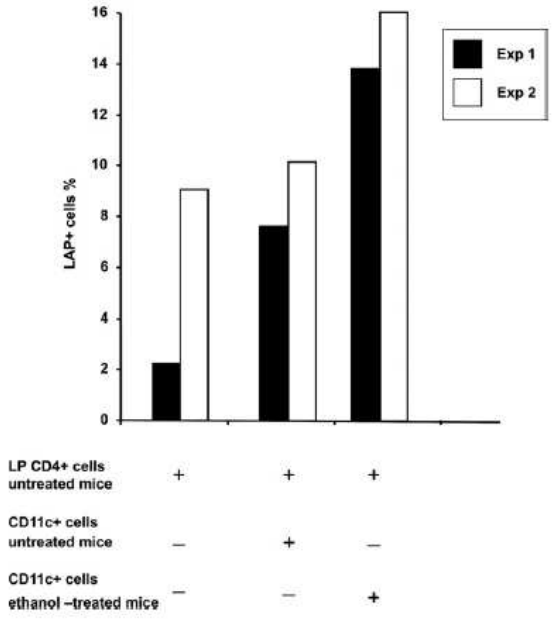

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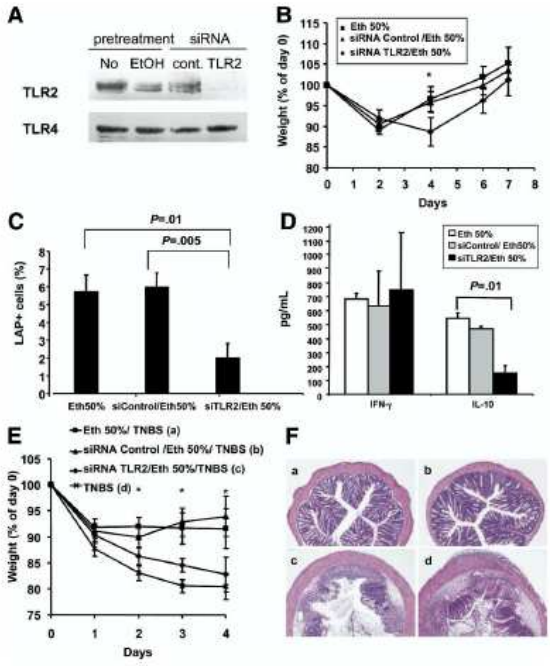

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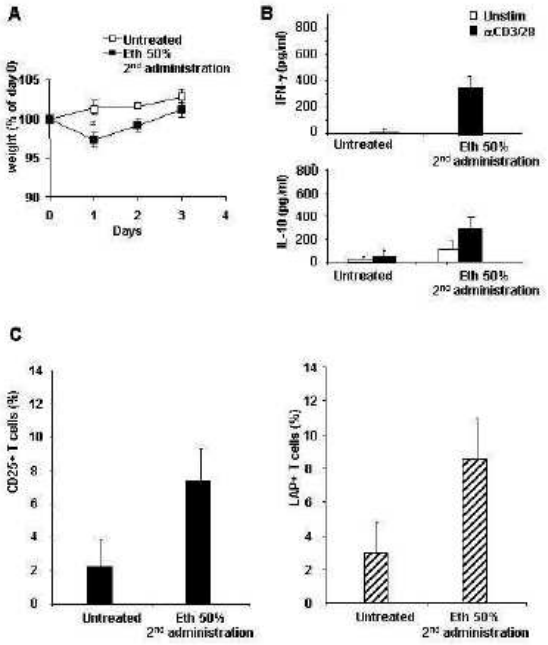
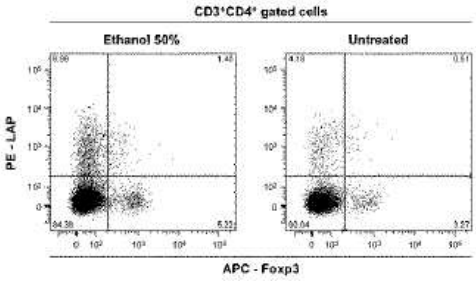


Supplementary Figure 1



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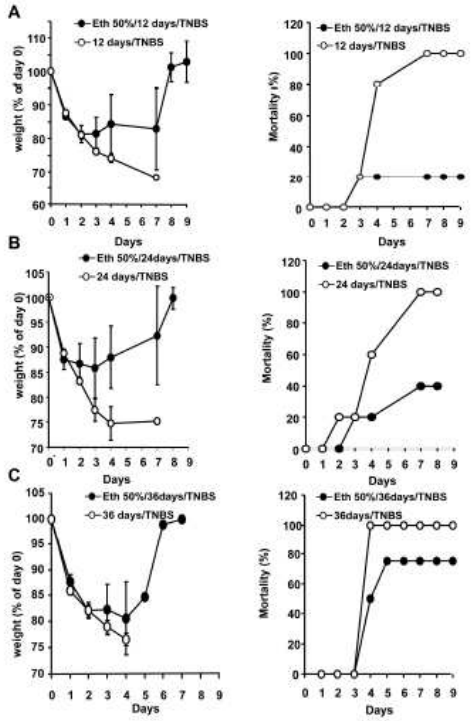
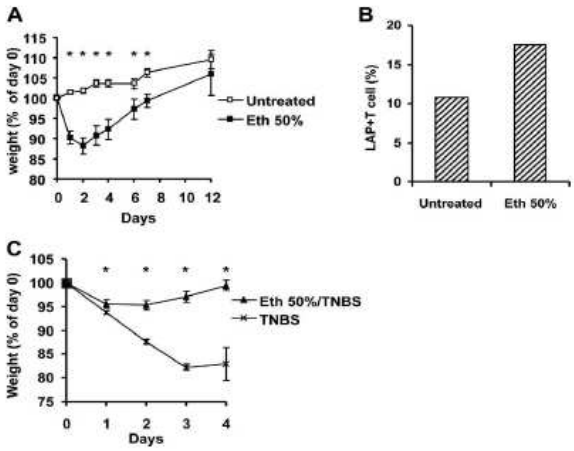


Supplementary Figure 3



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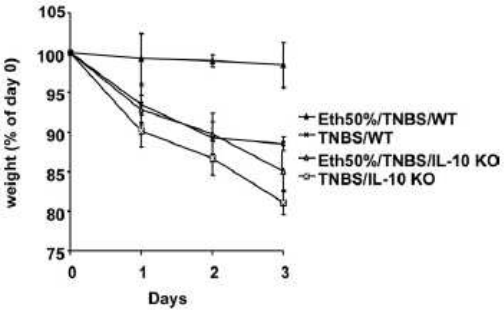

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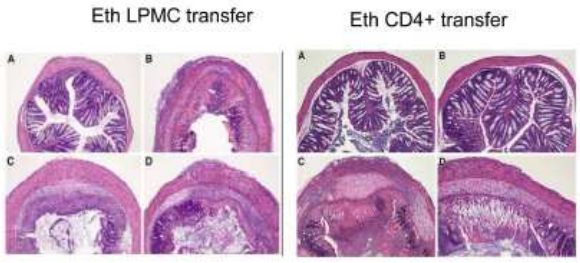

Supplementary Figure 6

Nod2 Deficiency is Associated with an Increased Mucosal Immunoregulatory Response to Commensal Microorganisms

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Antonello Amendola [1*], Alessia Butera[1*],Massimo Sanchez [2], Warren Strober [3], Monica Boirivant [1]

[1] Immune-Mediated Diseases Section, Department of Infectious, Parasitic, and Immune-Mediated Diseases, [2] Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy; [3]Mucosal Immunity Section, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland

*A.A and A.B. contributed equally to the study

Correspondence to: Monica Boirivant, MD, Immune- Mediated Diseases Section, Department of Infectious, Parasitic, and Immune-Mediated Diseases, Istituto Superiore di Sanità, Viale R. Elena, 299, 00161 Rome, Italy. e-mail: monica.boirivant@iss.it;

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Abstract

Based on previous studies demonstrating that a breach of the colonic epithelial barrier is associated with a microbiota-dependent increase in LP regulatory cells, we investigated if the lack of spontaneous intestinal inflammation observed in *Nod2-/-* mice was due to enhanced intestinal regulatory function. We found that the LP CD4+ T cell population of *Nod2-/-* mice contains an increased percentage of CD4+ regulatory T cells bearing TGF-β/latency peptide (LP CD4+LAP+ T cells) both under baseline conditions and following an intentional breach of the colonic barrier induced by ethanol administration. In addition, we found that *Nod2-/-* mice manifest decreased severity of TNBS-colitis and that TNBS-colitis in *Nod2-/- or Nod2+/+* mice is ameliorated by adoptive transfer of LP cells from ethanol-treated mice before, but not after, depletion of LAP+ T cells. This increased regulatory T cell response in *Nod2-/-* mice could explain why *NOD2* polymorphisms in humans are not in themselves sufficient to establish inflammatory lesions.

Introduction

NOD2 (nucleotide-binding oligomerization domain 2) is a member of the NLR (NOD, leucine-rich repeat (LRR)-containing protein) family of intracellular microbial sensors that has gained prominence because polymorphisms in the *CARD15* gene encoding this protein is the single most important genetic risk factor in Crohn's disease [1-4]. The NOD2 LRR sensor recognizes muramyl dipeptide (MDP), a component of the peptidoglycan present in the bacterial cell wall and thus NOD2 is likely to be an innate immune factor that participates in the control of organisms that enter the lamina propria. This has led to the view that abnormal Nod2 function associated with LRR polymorphisms leads to blunted clearance of such organisms and thus an inflammatory response mediated by innate immune functions unrelated to Nod2 [5, 6]. However, another view is based on evidence that Nod2 is a negative regulator of TLR signaling and its deficiency results in enhanced production of Th1 polarizing cytokines in the TLR-rich gut micro-environment [7]. Mice with *Nod2* deficiency have characteristics that bear on this question. For instance it has been shown that *Nod2-/-* mice exhibit increased CD4+ T cell IFN-γ production that is dependent on the presence of the intestinal microbiota and this, in turn, leads to increased bacterial translocation into the Peyer's patches (PP) and increased PP epithelial permeability due to induction of myosin light chain kinase, a factor that down-regulates tight junction integrity. Furthermore, such T cell-epithelial cell cross-talk under the control of TLR signaling, which is increased in *Nod2-/-* mice, but can be down-regulated in *Nod2+/+* mice by administration of MDP (Nod2 ligand). Thus it appears that bacterial translocation in *Nod2-/-* mice results from an absence of Nod2 regulation of TLR function [8, 9]. These findings favor the second hypothesis relating to polymorphic *NOD2* in Crohn's disease, namely that the latter leads to hyper-responsiveness [10].

Despite the above mentioned permeability changes, *Nod2-/-* mice do not develop overt intestinal inflammation, suggesting that Nod2 abnormalities are not sufficient to cause spontaneous and full-blown inflammatory lesions in themselves. Possibly relating to this, we previously demonstrated that a transient breach of the colonic epithelial barrier and an associated transient increase in the intestinal permeability is characterized by a microbiota-dependent increase in the generation of

regulatory cytokines and cells. In particular, such breaches were associated with the expansion Foxp3-negative CD4+ T cells expressing surface TGF-β associated with the latency associated peptide (LAP) (CD4+LAP+ T cells) that render mice resistant to the induction of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis [(11)]. Thus, the lack of spontaneous inflammation in mice with *Nod2* deficiency may be due to an enhanced mucosal regulatory response. To explore this hypothesis we investigated the mucosal regulatory response of mice with *Nod2* deficiency following a breach of the colonic barrier. We found that the lamina propria of *Nod2-/-* mice, when compared to *Nod2*+/+ (WT) mice, contains an increased percentage CD4+ T cells that are CD4+LAP+ regulatory T cells; furthermore, we found using cell transfer studies that these regulatory cells are likely to be responsible for the decreased severity of TNBS-colitis observable in *Nod2-/-* mice . Thus, an increased regulatory T cell response to microbiota in *Nod2-/-* mice could indeed explain why *NOD2* polymorphisms in humans are not sufficient to establish inflammatory lesions in the absence of other abnormalities.

Results

Nod2-/- mice exhibit increased colonic permeability associated with an expanded subpopulation of LP CD4+LAP+ T cells.

Since it has been reported that *Nod2-/-* mice show increased PP permeability and bacterial translocation [8], in initial studies we assessed colonic permeability and *in vivo* cytokine production in untreated *Nod2-/-* mice. As shown in Figure 1, we found that *Nod2-/-* mice exhibit a significant increase in baseline colonic permeability when compared with *Nod2+/+* mice as measured by uptake of intra-rectal FITC-dextran into the serum. Moreover, as also shown in Figure 1, this permeability change was associated with a significant increase in *in vivo* IL-12p70 production as measured by LP tissue content of this cytokine (as well as TNF-α, data not shown), suggesting that the permeability change had led to increased stimulation of LP macrophages and/or dendritic cells by innate stimuli derived from the intestinal microbiota. One might predict that this increased IL-12p70 production would be accompanied by increased LP T cell IFN-γ production, but, as shown in Figure 1, *in vivo* IFN-γ production in *Nod2-/-* mice was not increased as assessed by measurement of tissue content of this cytokine. It should be noted, however, that *Nod2-/-* LP T cells showed some evidence of enhanced Th1 differentiation in that stimulation of purified LPMC with anti-CD3/CD28 exhibited a significant increase in IFN-γ production *in vitro*.

To examine if the above lack of IFN-γ production *in vivo* could be due to down-regulation of LP T cell production of this cytokine we next determined if the *Nod2-/-* lamina propria contained increased regulatory factors. As shown in Figure 1, panel E flow cytometric studies revealed that *Nod2-/-* lamina propria CD4+ T cells populations contained a significantly increased percentage of LP CD4+LAP+ T cells and, moreover, *Nod2-/-* mice lamina propria exhibited a significant increase in tissue content of TGF-β, as compared to cells and tissues respectively from *Nod2+/+* mice (Figure 1, panel F). However, we did not observe an increase in the percentage of CD4+ Foxp3+ T cells within the LP CD4+ T cell population (*Nod2-/-*: 11.33 %± 1.42 vs. *Nod2+/+*: 10.68%±0.71;

mean± SE ; p=0.65). The above findings thus provide initial support for the possibility that the lack of increased IFN-γ secretion *in vivo* in *Nod2-/-* mice is in fact due to down-regulation of pro-inflammatory T cell differentiation by regulatory cells that do not express Foxp3.

Enhanced expression of CD4+LAP+ T Cells in Nod2-/- mice is dependent on an intact intestinal microbiota.

In previous studies it was shown that the intestinal microbiota acting through myosin light chain kinase (MLCK)-mediated effects on epithelial tight junctions was responsible for changes in the permeability of the Peyer's patch epithelium in *Nod2-/-* mice [(9)]. We reasoned that a similar mechanism may obtain with respect to the increased numbers of LP CD4+LAP+ T cells observed in *Nod2-/-* mice inasmuch as we have shown previously that induction of increased numbers of the latter cells in WT mice is also dependent on the presence of an intact intestinal microbiota [(11)]. In initial studies to examine this question we treated mice with ampicillin for 3 weeks and evaluated the frequency of LP CD4+LAP+ T cells in the treated mice (as well as in the untreated control mice). As shown in Figure 2, panel A, while ampicillin administration to *Nod2+/+* mice did not significantly affect the percentage of LP CD4+LAP+ T cells, administration to *Nod2-/-* mice was associated with a significant reduction of the percentage of LP CD4+LAP+ cells and at the end of the treatment period LP CD4+LAP+ levels in *Nod2-/-* mice were comparable to those in *Nod2+/+* mice. Thus, the increased numbers of LP CD4+LAP+ T cells in *Nod2-/-* mice is dependent on the presence of an intact intestinal microbiota.

With this information in hand, we next turned our attention to role of MLCK in the expansion of LP CD4+LAP+ T cells in *Nod2-/-* mice. To this end, we determined LP CD4+LAP+ T cell levels in *Nod2-/-* and *Nod2+/+* in mice pre-treated with an agent, ML-7, that has been shown to block the kinase activity of MLCK and thus inhibit its effect on intestinal permeability [(9)]. In initial studies shown in Figure 2, panel B, we first verified this effect by showing that ML-7

administration led to a reduction in serum concentration of intrarectally administered FITC-dextran in both *Nod2-/-* and *Nod2+/+* mice. Then, as shown in Figure 2, panel C, we showed that ML-7 administration led to a statistically significant reduction in the number of LP CD4+LAP+ T cells in *Nod2-/-* mice that was now not statistically different from that in *Nod2+/+*mice. Taken together, these studies suggest that an intact intestinal microbiota is necessary for the induction of increased LP CD4+LAP+ T cells in *Nod2-/-* mice and that such induction acts, at least in part, via MLCK effects on intestinal permeability.

The role of CD11c+ dendritic cells in LP CD4+LAP+ T cell expansion in Nod2-/- mice.

In previous studies we showed that CD11c+ dendritic cells (DCs) from ethanol-treated mice induced a greater increase in LP CD4+LAP+ T cells among co-cultured CD4+ T cells than an identical population of dendritic cells from untreated mice. This, taken with the observation that suppression of *in vivo* TLR2 responses by TLR2 siRNA administration had a down-regulatory effect on induction of LP CD4+LAP+ T cells, suggested that TLR-stimulated CD11c+ DCs were driving LP CD4+LAP+ T cell expansion [(11)]. Applying this information to a further examination of the role of CD11c+ DCs in the increased of LP CD4+LAP+ T cells in *Nod2-/-* mice, we co-cultured purified lamina propria CD11c+ cells from *Nod2-/-* and *Nod2+/+* with isolated LP CD4+ cells from *Nod2-/-* and *Nod2+/+* mice (at a 1:2 ratio) in various combinations and then assessed LAP+ expression after 48 h of culture. As shown in Figure 3, panel A, co-culture of CD11c+ DCs with CD4+ T cells from *Nod2-/-* mice, was associated with a larger increase in LAP+ T cells as compared to the increase in LAP+ T cells in co-cultures of CD11c+ DCs and CD4+T cells from *Nod2+/+* mice. Furthermore, increases seen in co-cultures of CD11c+ DCs from *Nod2-/-* mice and CD4+ T cells were equal regardless of the source of CD4+ T cells. These data thus suggest that DCs from *Nod2-/-* mice are more efficient in inducing the expansion of LAP+ T cells as compared to DCs from *Nod2+/+* mice.

In previous studies we also showed that TLR2 stimulation was responsible for the increased in LP CD4+LAP+ cell level observed after ethanol-induced increase of epithelial permeability(11). We performed co-culture experiments in the presence of the TLR2 stimulant Pam3CSK4 to further investigate the role of lamina propria dendritic cells in LP CD4+LAP+ cell expression. As shown in Figure 3, panel B, TLR2 stimulation of CD11c cells from *Nod2-/-* mice co-cultured with CD4+ cells from either *Nod2-/-* or *Nod2+/+* mice did not increase the percentage of LP CD4+LAP+ cells over the already increased percentage observed in unstimulated co-cultures. In contrast, TLR2 stimulation of CD11c cells from *Nod2+/+* mice co-cultured with CD4+ cells from *Nod2-/-* or *Nod2+/+* mice led to an increase in the percentage of LP CD4+LAP+ cells over the relatively low percentage observed in unstimulated co-cultures in two experiments.

Thus, CD11c dendritic cells not subjected to "*in vitro*" TLR2 stimulation from *Nod2-/-* mice are as efficient in inducing the expansion of LP CD4+LAP+ T cells due to sustained "*in vivo*" TLR2 stimulation as CD11c cells subjected to "*in vitro*" TLR2 stimulation from *Nod2+/+* mice. In view of the dependence of LP CD4+LAP+ T cells on the intestinal microbiota, these data also suggest that dendritic cells stimulated by TLR ligands in the intestinal microbiota are driving the increase in the number of these cells in the lamina propria of *Nod2-/-* mice.

Nod2-/- mice mount an enhanced regulatory T cell response and exhibit less inflammation upon challenge with i.r. ethanol.

In a previous study we showed that the number of LP CD4+LAP+ T cells in the colonic mucosa was augmented by administration of an agent, such as ethanol, that increases the permeability of the mucosal epithelial barrier. This and the fact that *Nod2-/-* mice display increased baseline mucosal permeability prompted us to explore further the lack of inflammation in the lamina propria of *Nod2-/-* mice and its possible relation to the presence of increased numbers of LP CD4+LAP+ regulatory T cells. To this end, we conducted studies in which *Nod2-/-* mice are subjected to intentional induction of increased mucosal permeability by ethanol administration. As shown in

Figure 4, panel A, intrarectal (i.r.) ethanol administration caused a transient increase in colonic permeability in both *Nod2-/-* and *Nod2+/+* mice. As shown in Figure 4, panel B, despite such increased permeability, *Nod2-/-* mice displayed significantly less weight loss as compared to *Nod2+/+* mice and, as shown in Figure 4, panel C, *Nod2-/-* mice exhibited little or no intestinal inflammation (colitis score: 0), whereas *Nod2+/+* mice exhibited mild intestinal inflammation (colitis score: 1-2). *In vivo* IL-12p70 and IFN-γ responses to ethanol administration provided parallel results; thus, as shown in Figure 4, panels D, while baseline IL-12p70 production was higher in *Nod2-/-* mice than in *Nod2+/+* mice, the latter mice exhibited a sharp rise in *in vivo* IL-12p70 production that led to a production level that exceeded that of *Nod2-/-* mice on the day of maximal permeability change, while *Nod2-/-* mice did not show any significant change in IL-12p70 tissue content after ethanol administration. As shown in Figure 4, panel E, a similar picture was observed for *in vivo* IFN-γ production although in this case baseline production of this cytokine were essentially the same in *Nod2-/-* and *Nod2+/+* mice. Corroborative results were obtained when we administered a second dose of 50% ethanol to the mice on day 4. Thus, as shown in Supplementary Figure 1, such secondary ethanol administration was again associated with less weight loss in *Nod2-/-* as compared to *Nod2+/+* mice.

The above difference between *Nod2-/-* and *Nod2+/+* mice in their response to i.r. ethanol administration could be the result from the fact that *Nod2-/-* mice mount a greater regulatory response. As shown in Figure 5, panel A and Supplementary Figure 2, evidence that this was indeed the case came from studies showing that *Nod2-/-* mice exhibited a greater increase in the percentage of LP CD4+LAP+ T cells among CD4+ LPMCs on the day of maximal permeability change and the relatively weak LP CD4+LAP+ T cell response of *Nod2+/+* mice was delayed. In both cases, however, the percentage of LP CD4+LAP+ T cells at day 3 did not differ from baseline levels of the untreated mice. Finally, as shown in Figure 5, panel B, *Nod2-/-* mice also mounted an earlier and higher *in vivo* TGF-β production response than *Nod2+/+* mice following

i.r. ethanol administration, although responses in both groups of mice were increased under these circumstances.

As previously reported [11], and as shown in the flow cytometric study depicted in Supplementary Figure 2, the majority of LP CD4+LAP+ T cells on day one after ethanol challenge were Foxp3-negative. However, as also shown in this figure, the percentage of LP CD4+LAP+Foxp3+ T cells among LPMC's also increased after ethanol administration at this time point. In addition, this increase was particularly evident 2-3 days after ethanol challenge: (*Nod2-/-* baseline: 11.71% ±0.74 vs. *Nod2-/-* day3: 16.27%±2.04 mean± SE p=0.01; *Nod2+/+* baseline: 9.93%±1.18 vs. day 2:16.03%±2.85 mean± SE p=0.02). Importantly, however, the increase in Foxp3+ T cells was equivalent in *Nod2-/-* and *Nod2+/+* mice, indicating that this did not explain the differences in degree of ethanol-induced inflammation and cytokine response between these groups.

The expansion of LP CD4+LAP+ T cells results in decreased severity of TNBS-colitis in Nod2-/- compared to Nod2+/+ mice.

To obtain further evidence that the increased percentage of LP CD4+LAP+ T cells of *Nod2-/-* mice before or after ethanol treatment is preventing the development of colitis in *Nod2-/-* mice we next conducted studies to determine if these cells affected the induction of TNBS-colitis, a colitis known to be modulated by LP CD4+LAP+ T cells and TGF-β[12]. To ensure valid results we conducted studies in *Nod2-/-* mice and control *Nod2+/+* mice that had been re-derived by embryo transfer into littermate foster mothers raised in the same SPF animal facility and in the same cage and then placed in separate cages prior to birth; thus, the mice had a similar origin and were reared in the same facility.

As shown in Figure 6, panels A and B, *Nod2-/-* mice administered intra-rectal TNBS (in 50% ethanol) to induce TNBS-colitis developed less severe colitis than *Nod2+/+* mice as shown by their lower weight loss and degree of colonic inflammation. In addition, as shown in Figure 6, analysis of anti-CD3/CD28-induced LPMC cytokine production *in vitro* showed that while IFN-γ production by LPMCs from *Nod2-/-* mice was significantly higher than that of LPMCs from *Nod2+/+* mice, IL-17 production by LPMCs from *Nod2-/-* mice was significantly lower than that of LPMCs from *Nod2+/+* mice and was equivalent to baseline (ethanol alone) IL-17 production. Taken together, these studies as well additional studies described below offer strong evidence that *Nod2-/- mice,* as compared to *Nod2+/+* mice, are resistant to induction of an induced colitis, TNBS-colitis.

To determine if the decreased severity of TNBS-colitis in *Nod2-/-* mice was attributable to the induction of LP CD4+LAP+ T cells we took advantage of our previous findings showing that LPMCs from mice treated with ethanol and thus with expanded LP CD4+LAP+ T cell populations can protect mice from TNBS-colitis upon adoptive transfer to the latter when cells are transferred before but not after depletion of LAP+ T cells [(11)]. In studies along these lines, we assessed the severity of TNBS-colitis in *Nod2-/-* and *Nod2+/+* mice after adoptive transfer of LPMC from *Nod2-/-* and *Nod2+/+* mice (respectively) that had been treated with ethanol (50%) one day prior to cell harvest and transfer. As shown in Figure 7, panels A-D, recipients of LPMC from ethanol-treated mice exhibited amelioration of colitis as assessed by weight loss and histological evaluation of colonic inflammation; however, the level of amelioration was greater in *Nod2-/-* recipients than in *Nod2+/+* recipients possibly due to the fact that ethanol-treated LPMC from *Nod2-/-* donors contains a higher level of LAP+ T cells. In contrast, as also shown in Figure 7, panels A-D, *Nod2-/-* recipients of LPMCs from ethanol-treated mice that had be subjected to LAP+ T cell depletion (See Methods) exhibited more severe colitis than mice treated with TNBS alone and equivalent to that observed in *Nod2+/+* treated with TNBS alone. Thus, the transferred LP CD4+LAP+ T cell depleted cell population derived from ethanol-treated mice contains cells that act as potent pro-

inflammatory T cells in the TNBS-treated recipient mice (see further studies of cytokine production by these cells below). Finally, *Nod2+/+* recipients of LPMCs from ethanol-treated mice that had been subjected to LAP+ T cell depletion exhibited the same level of colitis as mice treated with TNBS alone.

As shown in Figure 7, panels E-H, analysis of anti-CD3/CD28-induced *in vitro* cytokine production, by equivalent numbers of cell per culture, revealed that amelioration of TNBS-colitis observed in *Nod2-/-* and *Nod2+/+* recipients of LPMC (whether or not depleted of LAP+T cells) from ethanol-treated mice was not associated with changes in IFN-γ production. However, in both recipient groups, whereas transfer of undepleted LPMC was associated with non-significant reductions in IL-17 production, transfer of depleted LPMC was associated with significant increases in IL-17 production. These data thus indicate that increased severity of TNBS-colitis in recipients of LPMC depleted of LAP+ T cells correlates best with levels of IL-17 production per cell.

In complementary studies we performed adoptive transfer studies in which *Nod2+/+* mice with TNBS-colitis were transferred LPMCs from ethanol-treated *Nod2-/-* mice rather than *Nod2+/+* as in the experiments described above. As shown in Figure 8, transfer of LPMCs from *Nod2-/-* mice, was associated with amelioration of colitis, but in this case the level of amelioration was somewhat greater than with transfer of LPMCs from *Nod2+/+* mice. This is in keeping with the fact that ethanol treatment of *Nod2-/-* mice led to the generation of a greater number of LP CD4+LAP+ T cells than ethanol treatment of *Nod2-/-* mice (Figure 5) and that *Nod2+/+* mice recipient of *Nod2-/-* LAP- depleted LPMC show a TNBS colitis course comparable to the *Nod2+/+* mice treated with TNBS alone.

Overall, then, these adoptive transfer studies of TNBS-colitis are compatible with the view that the LP CD4+LAP+ T cell subpopulation generated by ethanol administration in *Nod2-/-* mice is capable of suppressing TNBS-colitis in both *Nod2-/-* and *Nod2+/+* mice and this suppression is greater than that obtained with a LP CD4+LAP+ T cell subpopulation generated by ethanol administration in *Nod2+/+* mice.

Influence of the endogenous Intestinal Microbiota on TNBS-Colitis in Nod2-/- Mice: co-housing studies

In studies described above it was shown that the generation of LP CD4+LAP+ regulatory cells was dependent on the presence of an intact intestinal microbiota. Thus, it seemed possible that *Nod2-/-* mice harbor a changed microbiota that affects the development of regulatory cells in the *Nod2*-deficient host. To examine this possibility we conducted co-housing studies in which *Nod2-/-* mice were maintained in the same cages with *Nod2+/+* mice or maintained in separate cages at weaning for four weeks before induction of TNBS-colitis. As shown in Figure 9, co-housed *Nod2-/-* mice exhibited more severe colitis and decreased LP CD4+LAP+ T cells than non-co-housed *Nod2-/-* mice. Reciprocal studies of *Nod2+/+* showed that *Nod2+/+* mice did not exhibit a change in baseline LP CD4+LAP+ cells (not shown). Thus, the *Nod2-/-* mice develop an intestinal microbiota that supports regulatory responses that contribute to the protection of mice from development of TNBS-colitis

Dextran sulfate sodium (DSS) colitis in Nod2-/- mice.

To determine if *Nod2*-deficient mice are protected from additional forms of experimental colitis we also assessed the severity of DSS-colitis in *Nod2-/-* and *Nod2+/+* mice. The mechanism of inflammation in DSS-colitis differs from that in TNBS-colitis in that it is dominated by a toxic

effect of DSS on epithelial cells followed by massive stimulation of innate cells (macrophages) by commensal gut bacteria and activation of the NLRP3 inflammasome [13]; thus, this colitis is less dependent on a T cell response as is TNBS-colitis. As shown in Supplementary Figure 3, in agreement with previous reports [14, 15], we observed that administration of low concentrations of DSS in drinking water led to more severe colitis in *Nod2-/-* mice than in *Nod2+/+* mice as shown by weight loss, myeloperoxidase activity and histology (the latter not shown). It was thus apparent that colitis dependent mainly on innate cell activation is under less regulation by LP CD4+LAP+ T cells than is colitis dependent mainly on T cells.

Discussion

In the present study we investigated the factors accounting for the observation that mice with *Nod2* deficiency do not exhibit significant spontaneous colonic inflammation despite the well-established role of *NOD2* polymorphisms as risk factors in Crohn's disease. We found that *Nod2-/-* mice manifest increased colonic permeability and heightened LP IL-12p70 production associated with little or no change in downstream LP IFN-γ production. The latter suggested the presence of a regulatory response and indeed the LP of *Nod2-/-* mice contained an increased subpopulation of LAP+ regulatory T cells among the lamina propria CD4+ cells that in subsequent studies was shown to be accentuated by intra-rectal ethanol administration and an accompanying increase mucosal permeability. We then showed that *Nod2-/-* mice are resistant to the development of TNBS-colitis and that such resistance is likely due to the presence of the LP CD4+LAP+ T cells. In particular, we found that adoptive transfer of LP T cells from ethanol-treated mice enhanced protection from TNBS-colitis whereas transfer of these cells following depletion of LAP+ T cells not only abolished such protection, it led to a level of colitis observed in *Nod2+/+* mice. Finally, we found that *Nod2-/-* mice develop a microbiota that favors the development of LP CD4+LAP+ regulatory cells and thus contributes to protection from induction of TNBS-colitis. These observations provide a strong rationale for why *Nod2* deficiency does not itself lead to overt intestinal inflammation as well as why humans with *NOD2* abnormalities require the presence of additional factors to manifest Crohn's disease.

Previous studies have provided evidence both in mice with *Nod2* deficiency and in humans with *NOD2* polymorphisms associated with Crohn's disease that NOD2 in myeloid cells functions as a negative regulator of TLR signaling and that such lack of "cross-regulation" of innate immune responses is a major reason why *NOD2* abnormalities may underlie the excessive mucosal immune responses that lead to Crohn's disease [7-9, 16]. In one of these previous studies as well as in the present study this consequence of *Nod2*-deficiency could also explain the fact that *Nod2-/-* mice exhibit high baseline (sub-inflammatory) mucosal cytokine production and increased activity of myosin light chain kinase, a factor that is the likely cause of the increased gut permeability in these

mice due to its effects on tight junctions. In addition, it could explain the dependence of these various abnormalities on the presence of the intestinal microbiota, the source of mucosal TLR responses and the increased capacity of lamina propria CD11c+ DCs from *Nod2-/-* mice to induce LP CD4+LAP+ T cells compared to similar DCs from *Nod2+/+* mice. On this basis the following sequence of events is a likely explanation for the increased LAP+ T cells response in *Nod2-/-* mice: 1) intestinal microbiota (via TLR stimulation) induce lamina propria DCs in *Nod2-/-* mice to produce sub-inflammatory amounts of cytokines that stimulate activation of MLCK; 2) MLCK causes increased intestinal permeability and further exposure of lamina propria DCs to microbiota TLR stimulation; 3) the DCs so-activated induce LP CD4+LAP+ regulatory cells. An additional factor bearing on the development of regulatory cells in *Nod2-/-* mice is that, as discussed in greater detail below, the Nod2 deficiency in *Nod2-/-* mice leads to the development of an intestinal microbiota with an increased capacity to induce the development of regulatory cells. Thus, the effects of *Nod2* deficiency on intestinal permeability is abetted by its effects on the intestinal microbiota.

The above analysis of the generation of regulatory cells in *Nod2*-deficient mice is compatible with the somewhat unexpected view that the consequences of *Nod2* dysfunction are two-fold. On the one hand, it leads to a tendency to mount increased TLR-induced responses that favor the development of inflammation and thus explain its role as a risk factor for Crohn's disease. On the other hand, as shown in these studies, it also leads to an increased TLR-induced regulatory response that establishes a resistance to inflammation. Which of these tendencies prevail is likely to depend on the nature, strength and persistence of the inflammatory stimulus. When the stimulus is relatively weak and self-limited as in the present study of TNBS-colitis in C57BL/6 mice the regulatory response prevails and one sees protection from colitis; in contrast, when, in the same mice, the stimulus is strong as in DSS-colitis and accompanied by massive breakdown of the epithelial barrier leading to invasion of the mucosa by commensal organisms, the inflammatory response prevails and one sees more inflammation. These different inflammatory conditions might model different environmental triggers of Crohn's disease and thus explain disease initiation and recrudescence in patients with NOD2 abnormalities.

LP CD4+LAP+ T cells are a unique regulatory cell population characterized by the presence of cell surface (LAP-associated) TGF-β and the absence of Foxp3 expression. As suggested by our previous observations in both mice and humans, this cell population tends to have a mucosal location and a size that is largely influenced by the prevailing microbiota [11, 12, 17]. Thus, we showed in a murine model of experimental colitis [12] and in humans with ileal-pouch-anal anastomosis for ulcerative colitis [17], that oral administration of a mixture of probiotics was able to expand lamina propria CD4+LAP+ T cells, while treatment of mice with oral antibiotics prevents their expansion [11]. The regulatory activity of LP CD4+LAP+ T cells has been highlighted in studies showing that they ameliorate colitis in both the cell transfer [18] and TNBS-colitis models [11, 12] and has, in addition, been recently confirmed in a model of murine asthmatic lung inflammation [19]. Finally, the regulatory activity of peripheral CD4+LAP+ T cells (possibly derived from the mucosal surface) has also been recently shown in humans [20]. The results of these various studies of LP CD4+LAP+ T cells, taken in conjunction with those in the present study, establish that this cell population has an important role in maintaining immune homeostasis in the microbiota-rich environments of the mucosal surfaces.

In the present study we observed a selective expansion of CD4+LAP+Foxp3- T cells in *Nod2-/-* mice as compared to *Nod2+/+* T cells but no comparable expansion of the CD4+Foxp3+ cell population. However, the latter population did expand equally in *Nod2-/-* and *Nod2+/+* mice in response to a transient increase in intestinal permeability induced by intra-rectal ethanol administration. These results emphasize that CD4+Foxp3+ cells are distinct from CD4+LAP+ T cell population in this context and do not underlie the intestinal regulatory response associated with *Nod2* deficiency. Nevertheless, these two cell populations are likely to be related on some level given the fact that membrane-bound TGF-β bound to LAP is present on both types of cells and, as such, is likely to be involved in their respective suppressive activities [21]. In addition, it has recently been shown that Foxp3 induces surface TGF-β/LAP expression and that TGF-β induces TGF-β/LAP expression in Foxp3- cells [22]. This, plus the well known fact that TGF-β induces Foxp3, suggests a broad (if as yet incompletely defined) inter-relation between TGF-β, Foxp3 and

surface TGF-β/LAP expression. This proposed inter-relationship, however, does not necessarily imply that CD4+LAP+Foxp3- T cells and CD4+Foxp3+ cells belong to the same cell lineage or share the same set of inductive factors [23]. The latter is evident from the present study in that the induction of CD4+LAP+Foxp3- cells appears to require special conditions that include the presence of high levels of TGF-β as well as dendritic cells producing a possibly unique mixture of cytokines resulting from the strong TLR signaling present in the lamina propria in *Nod2-/-* mice. This view is at least partially supported by the observation that the kinetics of LP CD4+LAP+Foxp3- T cell expansion observed in our study parallels the kinetics of TGF-β production and from previous evidence that the *in vivo* generation of CD4+ LAP+ Foxp3- cells is dependent on both TGF-β and IL-10 [19].

As indicated above, in the present study we observed that *Nod2-/-* mice exhibit less severe TNBS-colitis when compared to *Nod2+/+* mice. This finding correlated with the fact that in adoptive transfer studies we showed that LPMC from *Nod2-/-* mice donor mice had a greater capacity to ameliorate colitis than cells from *Nod2+/+* donor mice. In addition, we showed that this effect reflected the greater number of LP CD4+LAP+ T cells in the *Nod2-/-* cell population as deletion of these cells from the transferred cell population abolished the protective effect of cell transfer.

Thus, these transfer studies provided a mechanistic basis for the decreased severity of colitis in *Nod2-/-* mice. Somewhat unexpectedly, on a per cell basis, the decreased inflammation of TNBS-colitis in *Nod2-/-* mice was associated with decreased LPMC IL-17 and increased IFN-γ production as compared to *Nod2+/+* mice. Furthermore, *Nod2-/-* recipients of adoptively transferred LAP+ T cell-depleted LPMC exhibited a cytokine response comparable to that of *Nod2+/+* mice that was characterized by increased LPMC production of IL-17 but no change in IFN-γ production. These data suggesting that IL-17 production plays a more important role in the pathogenesis of TNBS-colitis than IFN-γ in C57BL/6 mice are in agreement with a previous report wherein it was shown that while such colitis was marked by increased *in vivo* IL-17 and IFN-γ production, less severe colitis was observed in IL-17R-deficient mice despite the presence of increased IFN-γ tissue levels

[24]. In addition, the increase in IL-17 production in recipients of CD4+LAP+ -depleted cells suggests that the CD4+LAP+ T cells might be acting, in part, via regulation of Th17 cells.

The decreased severity of TNBS-colitis in *Nod2*-/- mice observed in our study is in contrast with previous studies in which an increased severity of colitis in *Nod2*-/- mice compared to *Nod2*+/+ mice was reported [8, 25]. This may have been due to differences in the intestinal microbiota acquired by mice in the various mouse-housing facilities utilized since it is known that at least IL-17 production can be influenced by nature of the resident microbiota [26]. Thus, it is possible that *Nod2*-deficient mice is various facilities acquire somewhat unique sets of microbiota more or less capable of inducing pro-inflammatory effector cells and anti-inflammatory regulatory T cells that then serve as environmental stimuli leading to different *Nod2*-deficiency outcomes as discussed above. Also bearing on this issue are recent studies that have shown that changes in the intestinal microbiota are associated with *Nod2* deficiency [27, 28]. Indeed, our studies corroborate this finding in that we have found in co-housing studies that *Nod2-/-* mice reared together with *Nod2*+/+ mice post-weaning developed less LP CD4+LAP+ cells and more severe TNBS-colitis than *Nod2*+/+ mice reared independently. It was thus evident that the microbiota in *Nod2-/-* mice was distinct from that in *Nod2*+/+ mice in that it had a greater capacity to induce regulatory cells. This implies that the Nod2-deficiency in *Nod2-/-* mice operates in two ways to favor regulatory cell development: 1) it leads to permeability changes that increase exposure of dendritic cells to factors such as TLR ligands that induce such development and 2) it creates a milieu that permits changes in the microbiota that support such development. At this point the nature of these latter changes are quite undefined although we would speculate that they involve selective expansion of organisms similar to previously described inducers of regulatory cells such as members of the *Clostridium* bacterial family [29]. Delineation of this possibility is clearly an area of potentially fruitful future work.

In summary, data from the present study suggest that the increased intestinal permeability observed in *Nod2-/-* mice is associated with an increased homeostatic regulatory response mediated by LP CD4+LAP+ T cells. They thus suggest that factors influencing this regulatory response (or

possibly over-riding this response) might be necessary to induce a full-blown inflammation in the presence of *Nod2* deficiency. This conclusion is relevant to IBD in humans where increased intestinal permeability [30, 31] and an altered microbiome [32, 33] have been reported to be associated with *NOD2* polymorphisms in the absence of inflammation.

Materials and Methods

Mice

Nod2+/+ and *Nod2-/-* mice on C57BL/6 background were kindly donated by Peter J Murray, Department of Infectious Diseases and the Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA [34]. At the Istituto Superiore di Sanità *Nod2-/-* mice and control *Nod2+/+* mice had been re-derived by embryo transfer into littermate foster mothers raised in the same SPF animal facility and in the same cage. *Nod2-/-* and *Nod2+/+* mice were then maintained in SPF conditions in the animal facility at the Istituto Superiore di Sanità, Roma, Italy. All mice were housed with free access to food and water. Pathogen-free conditions were monitored every 6 months in accordance with the full set of FELASA high standards recommendations. All animal studies were approved by the Animal Care and Use Committee of the Istituto Superiore di Sanità.

Study design

All studies were performed in 7-8 week old male mice. In studies of ethanol effects on regulatory T cell expression mice were administered 50% ethanol, via a 3.5 F catheter inserted into the rectum as previously described [11]; subsequently, mouse weights and histological appearance of the colonic tissue was determined at various time points. In studies on the effects of myosin light chain kinase (MLCK) inhibition, mice were administered intraperitoneal injections ML-7 (2mg/kg; Sigma-Aldrich, Milan, Italy), a MLCK inhibitor, twice daily for 4 days [9]. In studies on the effect of antibiotic administration, mice were administered ampicillin (Pfizer Italia srl, Latina, Italy) 200µg/100µl distilled water by oral gavage for 14-21 days; control mice received water only. In studies of TNBS-colitis, mice were administered TNBS 3.5 mg (Sigma-Aldrich) delivered in 150µl of 50% ethanol per rectum as previously described [11].

Intestinal permeability studies

Untreated mice or ML-7 treated mice were administered with a dose of 2mg/10g body weight of FITC-conjugated dextran dissolved in purified water (4000 mw, Sigma-Aldrich, Milan, Italy) per rectum. Whole blood was obtained via eye bleed one hour after FITC dextran administration. Blood samples were centrifuged at 3000 rpm to obtain sera; the latter was then analyzed by fluorometry using a Victor3™ (485/535nm, gain 1480) plate reader (Perkin-Elmer, Waltham, MA). Mice administered 50% ethanol per rectum were subsequently administered daily doses of FITC-conjugated dextran dissolved in purified water per rectum and whole blood was obtained via eye bleed one hour after FITC dextran administration.

Adoptive transfer of LPMC

LPMC were isolated from colons of *Nod2+/+* and *Nod2-/-* ethanol-treated mice 1 day after ethanol administration. LPMC (4.0×10^5) were then injected i.v. into the tail vein of *Nod2+/+* and *Nod2-/-* mice, respectively. In some experiments, additional mice of each group were administered LPMC depleted of LAP+ T cells by cell sorting after staining cells with anti-mouse CD4-PerCP (RM4-5 BD Pharmingen, S. José, CA) and biotinylated goat anti-LAP (R&D Systems, Minneapolis, MN) -streptavidin APC using a FACS-ARIA. TNBS 3.5 mg was administered per rectum to the recipient mice 5 days after the LPMC transfer as previously described [(11)]. The occurrence and course of colitis in the different groups of animals was evaluated as described in the text.

Histological assessment of colitis

Tissues removed from mice at indicated times of death were fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) and then embedded in paraffin, cut into tissue sections, and stained with H&E. Stained sections were examined for evidence of colitis by a pathologist (ISTOVET di Luca

Crippa & C. S.A.S., Besana in Brianza (MB), Italy) and scored using previously reported criteria [11]

Protein extraction

Mouse colonic specimens were rinsed with cold PBS, blotted dry, and immediately frozen in liquid nitrogen for protein extraction .Total protein extracts were prepared as previously described [35]. Briefly, snap frozen mucosal samples were homogenized in buffer containing 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM EDTA, and 0.2% Nonidet, supplemented with 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethanesulphonyl fluoride, 1mM sodium orthovanadate, 1mM sodium fluoride (all reagents were from Sigma)

Isolation and Culture of LP Mononuclear Cells (LPMC)

LPMC were isolated from freshly obtained colonic specimens using a modification of the method described by van der Heijden and Stok [36]. Isolated LPMC were cultured at $1x10^6$ cells /ml in 48-well plates (Costar, Corning Incorporated life sciences, Tewksbury, MA) coated or uncoated with murine anti-CD3e antibody (clone 145-2C11; BD Pharmingen) and containing X-Vivo medium (Biowhittaker, Walkersville, MD) supplemented with 1 μg/ml of soluble anti-CD28 antibody (clone 37.51; BD Pharmingen), as previously described [11] . After 48 hours culture supernatants were harvested and stored at -80°C for future ELISA assay.

In vitro Stimulation of CD4+ LP T Cells by CD11c+ LP DCs

LPMC cells obtained from untreated mice were subjected to magnetic bead sorting using CD11c microbeads or a CD4+ T cell Isolation Kit II (Miltenyi Biotech, Auburn, CA) following the manufacturer's instructions. The purity of LPMC CD11c+ and CD4+ cells were > 90 % and >95

%, respectively as assessed by immunofluorescence staining. $2x10^5$ /ml of the CD4+ cells obtained from *Nod2-/-* or *Nod2+/+* untreated mice were co-cultured with $1x10^5$/ml CD11c+ cells obtained from *Nod2-/-* or *Nod2+/+* untreated mice in the presence or in the absence of 500ng/ml Pam3CSK4 (InvivoGen, San Diego, CA). At the end of 48 h culture, the cells were collected, washed and stained for LAP expression by flow cytometry.

LPMC Immunofluorescence staining

Freshly isolated and washed LPMC were subjected to Fc block with anti-CD16/CD32 (BD-Pharmingen) and then stained with biotinylated goat anti-LAP (R&D Systems) or biotinylated normal goat IgG (R&D Systems).

After 30' incubation, cells were washed and incubated with streptavidin-APC. The cells were then incubated with PerCP-anti-mouse-CD3 (145-2C11, BD Pharmingen) and APC- Cy7 anti-mouse CD4 (GK1.5, BD Pharmingen), for an additional 30'. In some experiments, intracellular Foxp3 expression was evaluated using APC-anti-mouse/rat Foxp3 staining set (eBioscience, San Diego, CA, USA) following the manufacturer‘s protocol. The cells were then washed twice, and the percentage of fluorescent cells was quantified using a FACSAria (BD Biosciences)

ELISA

Cytokine concentrations in protein extracts and cell supernatants were determined by commercially available specific ELISA kits. IFN-γ: BD-OptEIA Set Mouse (BD Bioscience); IL-12p70: Quantikine ELISA (R&D Systems); IL-17: Quantikine ELISA (R&D Systems), IL-17: BD-OptEIA Set Mouse (BD Bioscience); mouse/rat/porcine/canine TGF-β1 quantikine (R&D systems) per the manufacturer's recommendations).

Statistical analysis

Assessment of statistical differences was determined by Student's t test or by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test when appropriate.

Supplementary Material is linked to the online version of the paper at http://www.nature.com/mi

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Figure Legends

Figure 1. *Nod2-/- mice exhibit increased colonic permeability and increased in vivo IL-12p70 production, associated with an increased LP subpopulation of CD4+LAP+ T cells and enhanced in vivo TGF-β production.*

A: measurement of colonic permeability by intrarectal administration of FITC- Dextran (see Methods). Data shown is derived from two separate experiments, each involving five mice/group; data represent mean±SE; B and C: *In vivo* IL-12p70 and IFN-γ production: cytokine content of protein extracted from lamina propria tissue was measured by ELISA; data represent mean±SE derived from 12 mice in each group; D: LPMCs isolated from colonic tissue were cultured for 72 h in the absence of stimulation (US) or in the presence of anti-CD3/28 stimulation, after which the culture supernatants were assayed for IFN-γ content by ELISA; data represent mean±SE derived from 10 mice in each group. Data were tested for statistical significance using Student's *t* test. E: Percentage of LAP+ cells among CD3+CD4+ gated LP cells; data represent mean±SE derived from 12 mice/group; F: *In vivo* TGF-β production: TGF-β content of protein extracted from lamina propria tissue was measured by ELISA. Data represent mean±SE derived from 12 mice/group. Data were tested for statistical significance using Student's t test.

Figure 2. *Effect of ampicillin treatment (A) and ML-7 treatment (B,C) on the fraction of LP CD4+LAP+ cells among CD4+ LP cells.*

A. Separate groups of mice were treated with ampicillin 200μg/100μl distilled water or 100μl distilled water alone (untreated) by oral gavage; for 14-21 days. Mice were sacrificed at 14 and 21 days of Ampicillin treatment, and at 21 days of water treatment, respectively. LP cells from colons isolated, stained for CD3, CD4 and LAP and analyzed by flow cytometry. Each column represent mean±SE * =P<0.05. *Nod2-/-* mice untreated (distilled water only) vs Ampicillin-treated 21 days by Student's *t* test. B. Separate groups of mice were treated for 4 days with ML-7 twice a day i.p

(see methods) . At the end of treatment mice were intrarectally administered FITC-Dextran (see methods). Columns represent mean±SE of three mice/ group. C. Separate groups of mice were treated for 4 days with ML-7 twice a day i.p (see methods). At the end of treatment mice were sacrificed, colons were collected and isolated LP cells stained for CD3, CD4 and LAP and analyzed by flow cytometry. Columns represent mean±SE of data from three different experiments. * P<0.05. *Nod2-/-* mice untreated vs ML-7 treated mice by Student's *t* test.

Figure 3. *Induction of LP CD4+LAP+ cells by CD11c DCs in vitro.*

A. Percentages of LAP^+ cells in purified lamina propria CD4+ T cell populations following co-culture for 48 hrs. with $CD11c^+$ DCs isolated from the lamina propria of *Nod2-/-*and *Nod2+/+* mice. Column represent mean±SE of data from two different experiments. B. Percentages of LAP^+ cells in purified lamina propria CD4+ T cell populations following co-culture for 48 h. with $CD11c^+$ DCs isolated from the lamina propria of *Nod2-/-* and *Nod2+/+* mice in the presence or in the absence of Pam3CSK4. Column represent mean±SE of data from two different experiments.

Figure 4. *Nod2-/- mice exhibit increased permeability but decreased inflammation vs. Nod2+/+ mice upon intra-rectal ethanol administration.*

A. Colonic permeability in *Nod2-/-* and *Nod2+/+* mice after administration of intra-rectal 50% ethanol measured by serum FITC-dextran uptake (See Methods); data represent mean ±SE derived from two different experiments in which three mice/group were evaluated *=P<0.05 day 1 vs untreated; B. Mouse body weight changes after intra-rectal ethanol administration; each point represents mean±SE of daily weights expressed as percentage of day 0 weight; data represent pooled values derived from three different experiments in which five mice/group was studied. * P<0.01 *Nod2-/-* vs. *Nod2 +/+*; C. Representative microscopic appearance of colons of mice administered ethanol and sacrificed at the time-points shown ; H&E staining 40x magnification. *Nod2-/-* and *Nod2+/+* untreated and day 3: normal colonic architecture/patterns. *Nod2+/+* day2:

Presence of mild submucosal edema and mild inflammatory infiltration. *Nod2-/-* day 2: normal colonic architecture/pattern; D: *In vivo* IL-12p70 production after 50% ethanol administration. Each point represents mean± SE of pooled values derived from two experiments in which 5 mice/group were evaluated at each time point; * P<0.05 *Nod2+/+* day1 vs. untreated mice. **P<0.05 *Nod2-/-* vs. *Nod2+/+* untreated mice. E: IFN-γ tissue content at different days after ethanol administration. Each point represents mean± SE as described in D;* P<0.05 *Nod2+/+* day1 vs. untreated. Data were tested for statistical significance using Student's *t* test.

Figure 5. *Effect of ethanol administration on CD4+LAP+ T cells*

A. Percentage of LAP+ T cells among CD3+CD4+ gated LP cells after ethanol administration. Each point represents mean± SE of pooled values derived from three experiments in which 5 mice/group/ were evaluated; * P<0.05 *Nod2-/-* day 1 vs. untreated; * P<0.05 *Nod2+/+* day 2 vs. untreated; ** P<0.05 *Nod2-/-* vs. *Nod2+/+* untreated and day 1; B. *In vivo* TGF-β production after ethanol administration. Each point represents mean± SE as described in A. * P<0.05 day1-2 vs. untreated *Nod2-/-* mice; * P<0.05 day 1-2-3 vs. untreated Nod2+/+ mice; **P<0.05 untreated and day 1 *Nod2-/-* vs. *Nod2+/+* mice. Data were tested for statistical significance using Student's *t* test.

Figure 6. *Nod2-/- mice exhibit less severe TNB-colitis compared with Nod2+/+ mice.*

Mice were administered intra-rectal ethanol/TNBS to induce TNBS-colitis or ethanol alone. A. Body weight changes; Each point represents mean ±SE weight at indicated times expressed as percentage of day 0 weight and derived from three different experiments in which five mice/group were evaluated; * P<0.05 ethanol/TNBS-treated *Nod2-/-* vs. *Nod2+/+* ; B. histological appearance of representative colonic tissue of TNBS-treated mice sacrificed at day three after TNBS administration; H.&E. staining, 40x magnification: a, *Nod2+/+*: severe, diffuse ulcerative colitis with dense transmural inflammatory cell infiltration of the submucosa, the smooth muscle and the

serosa layers. b, *Nod2-/-* mild, non-ulcerative colitis with mild submucosal edema and mild inflammatory cell infiltration; Colitis score *P<0.05 ethanol/TNBS-treated *Nod2-/-* vs. *Nod2* +/+ by Student's *t* test. C. and D. *in vitro* IFN-γ and IL-17 production by unstimulated and anti-CD3/CD28-stimulated LPMC; data represent mean±SE of pooled values derived from three different experiments in which five mice/group were evaluated . Data were tested for statistical significance using one way analysis of variance with Bonferroni's multiple comparison test.

Figure 7. *Adoptive transfer of CD4+LAP+ T cells ameliorates severity of TNBS-colitis*

Mice were administered intra-rectal ethanol/TNBS to induce TNBS-colitis alone or TNBS-colitis in adoptively transferred LPMCs or LAP+ T cell-depleted LPMCs mice. A and B. Body weight changes; each point represents mean ±SE weight at indicated times expressed as percentage of day 0 weight and derived from an experiment in which six mice/group were evaluated; * P<0.05 TNBS-colitis in *Nod2-/-* recipients of LAP+ T cell-depleted LPMC vs. recipients of LPMC and *Nod2-/-* mice with TNBS-colitis alone; C: histological appearance of representative colons of different groups of *Nod2-/-* mice sacrificed at day 3 after TNBS administration; H&E staining, 40x magnification: a, mild submucosal edema with mild inflammatory cell infiltration; b, moderate ulcerative colitis with moderate submucosal edema and inflammatory cell infiltration; c, normal colonic architecture/patterns; Colitis score: * P<0.05 TNBS-colitis in *Nod2-/-* recipients of LAP-depleted LPMC vs. recipients of LPMC and *Nod2-/-* mice with TNBS-colitis alone. D. histological appearance of representative colons of different groups of *Nod2+/+* mice sacrificed at day 3 after TNBS administration; H&E staining, 40x magnification: a, severe, diffuse, transmural ulcerative colitis with severe, submucosal inflammatory cell infiltration also involving the smooth muscle and serosa layers; b, marked ulcerative colitis with moderate submucosal edema and inflammatory cell infiltration; c**,** moderate ulcerative colitis with moderate submucosal edema and inflammatory cell infiltration. E-H: "*in vitro*" IFN-γ and IL-17 production by isolated LPMC. Data represent cumulative mean±SE from 6 mice /group. Data were tested for statistical significance using one way analysis of variance with Bonferroni's multiple comparison test.

Figure 8. Mice were administered intra-rectal ethanol/TNBS to induce TNBS-colitis in untreated *Nod2*+/+ mice or in *Nod2*+/+ mice adoptively transferred LPMCs or LAP- depleted LPMCs from ethanol-treated *Nod2*-/- mice. A: Body weight changes; each point represents mean ±SE weight at indicated times expressed as percentage of initial weight; these data were from 2 experiments with eight mice/group except for LAP-depleted LPMC recipient group in which 5 mice were evaluated; *P<0.05 TNBS-colitis in *Nod2*+/+ recipients of *Nod2*-/- LPMC vs. *Nod2*+/+ mice with TNBS-colitis and *Nod2*+/+ mice recipient of LAP -depleted *Nod2*-/- LPMC B: histological appearance of representative colons of different groups of mice sacrificed at day 3 after TNBS administration; H&E staining, 40x magnification: a, severe, diffuse, transmural ulcerative acute colitis with severe submucosal inflammatory infiltration deeply involving the smooth muscle and serosa layers; b, moderate submucosal oedema and moderate inflammatory infiltration ; c, severe, diffuse, transmural ulcerative acute colitis with severe submucosal inflammatory infiltration deeply involving the smooth muscle and serosa layers ;d, normal architecture/patterns with small lymphoid nodules. Colitis score: ;*P<0.05 TNBS-colitis in *Nod2*+/+ recipients of *Nod2*-/- LPMC vs. *Nod2*+/+ mice with TNBS-colitis and *Nod2*+/+ mice recipient of LAP-depleted *Nod2*-/- LPMC. Data were tested for statistical significance using one way analysis of variance with Bonferroni's multiple comparison test.

Figure 9. Nod2-/- mice were either single-housed (n=5) or co-housed at a 1:1 ratio with Nod2+/+ mice (n=5) on the same diet for a 4-week period. A: % of LP CD4+LAP+ cells in the single-housed or co-housed mice. *P<0.05 by Student's *t* test. B: Body weight changes; each point represents mean ±SE weight at indicated times expressed as percentage of day 0 weight. *P<0.05 by Student's *t* test. C: Histological appearance of representative colons of mice sacrificed at day 3 after TNBS administration; H&E staining, 40x magnification. Colitis score: * P<0.05 by Student's t test

Supplementary Figure 1. Mice were administered ethanol 50% intrarectally at day 0 and at day 4 after the 1st administration. A: Body weight changes; each point represents mean ±SE weight at indicated times expressed as percentage of day 0 weight and derived from an experiment in which five mice/group were evaluated. B: % of LP LAP+ cells in CD3+CD4+ gated LPMC observed in mice sacrificed before and at day 4 after the 1st ethanol administration. Data represent values obtained from pooled LPMC isolated from 3 additional mice at day 0 and 4.

Supplementary Figure 2. Representative dot plots of LPMC stained for LAP and Foxp3 (% in CD3+CD4+ gated LP cells) isolated from colons of *Nod2-/-* and *Nod2+/+* mice treated or not with ethanol 50% i.r.

Supplementary Figure 3. Mice received dextran sulfate sodium (M.W.36, 000-50,000) (DSS) 2% (w/v) in their drinking water for 7 days. A: Body weight changes; each point represents mean ±SE weight at indicated times expressed as percentage of initial weight and derived from an experiment in which five mice/group were evaluated. * $P<0.05$ *Nod2-/-* vs *Nod2+/+* mice at indicated times after DSS administration by Student's *t* test. B: Mice were sacrificed at day 12, colons were collected and myeloperoxidase activity (MPO) was evaluated. * $P<0.05$ *Nod2-/-* vs *Nod2+/+* mice by Student's *t* test.

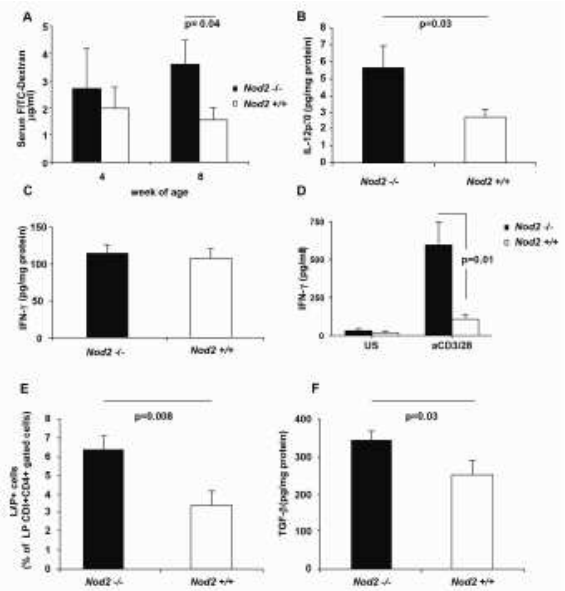
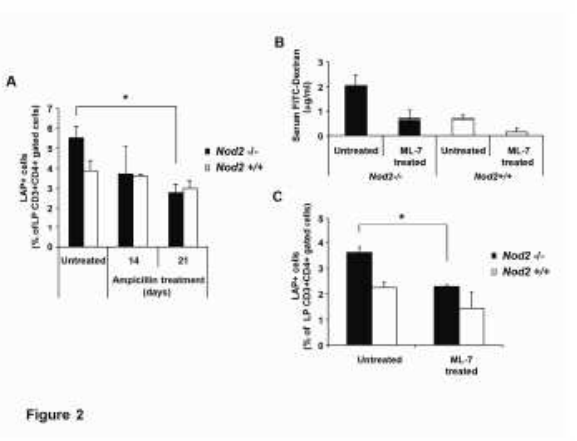


Figure 1



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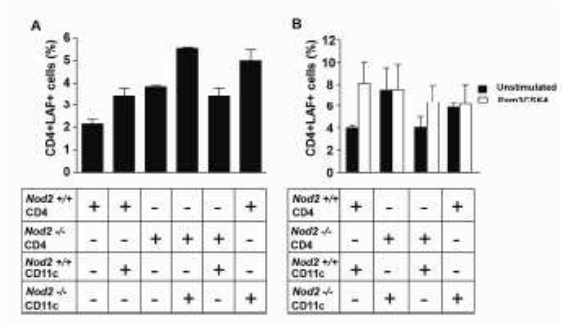

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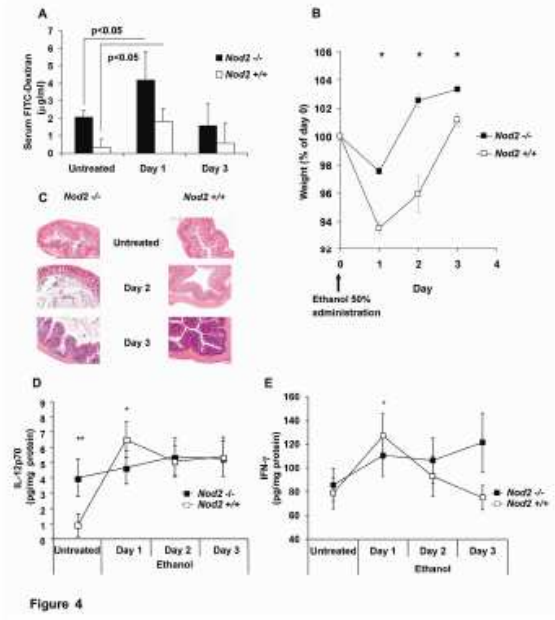

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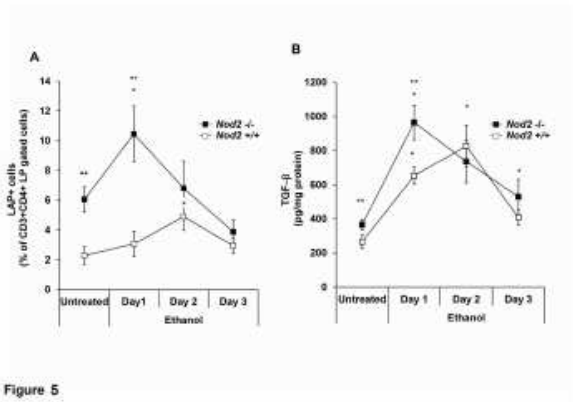

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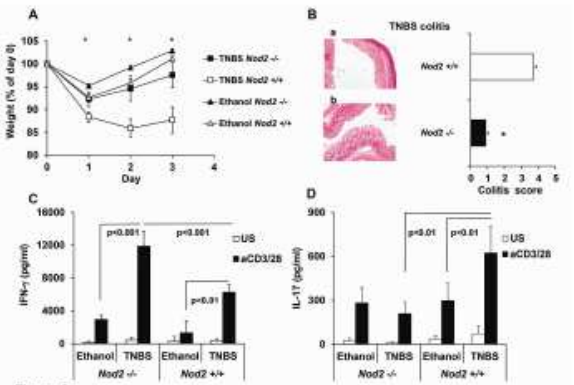

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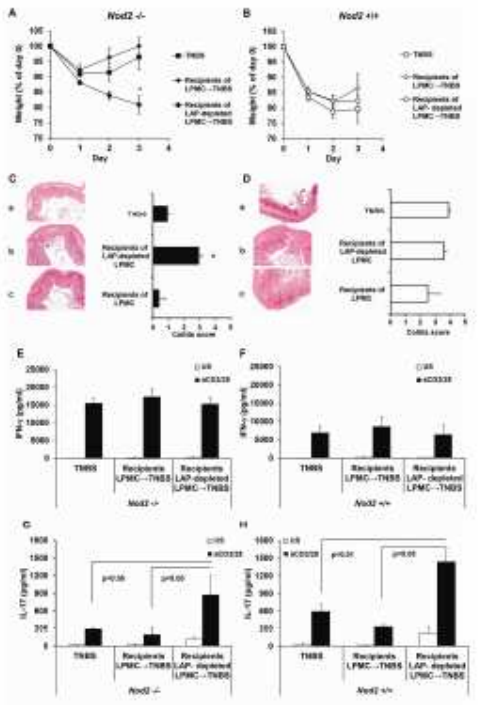

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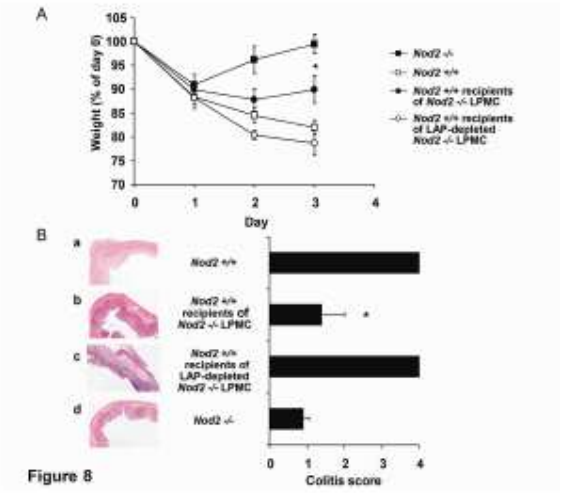

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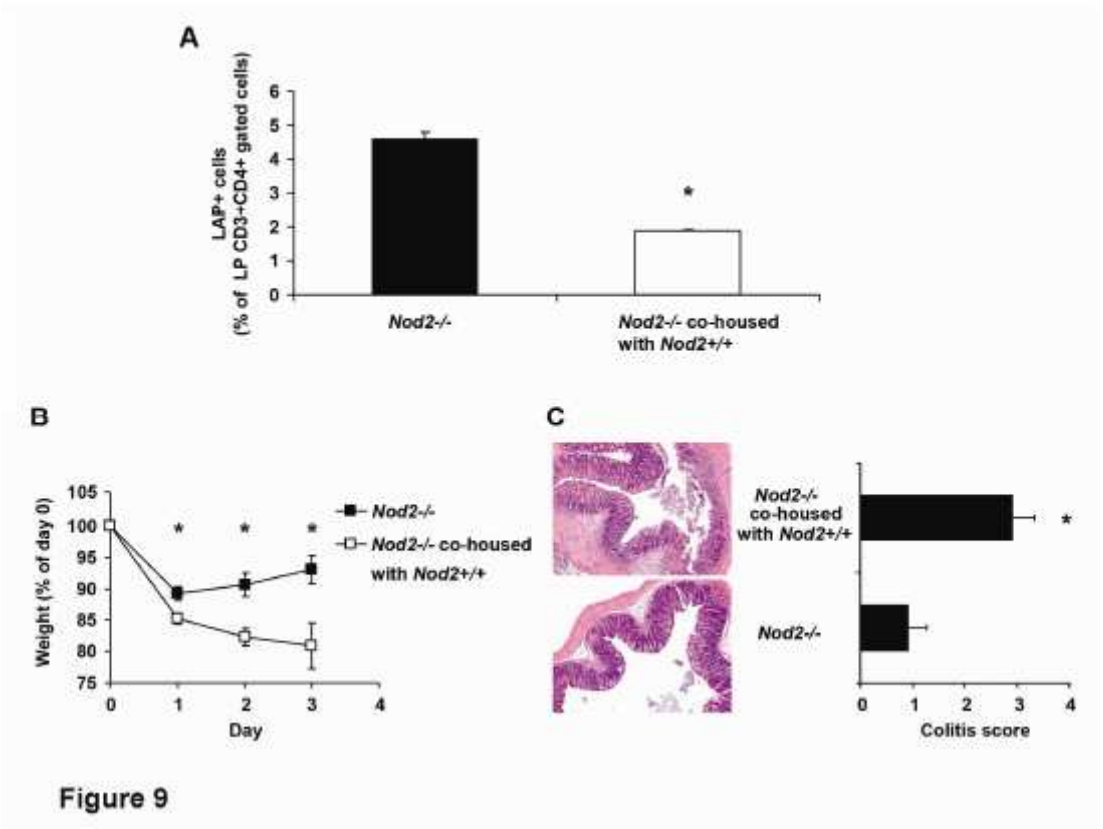

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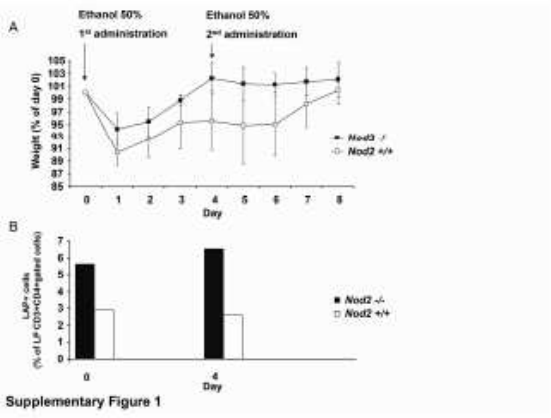
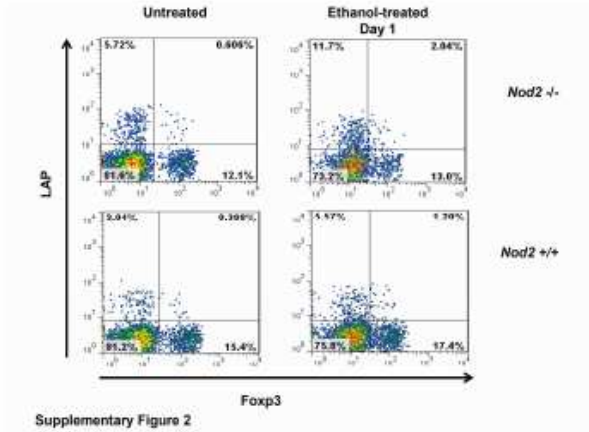
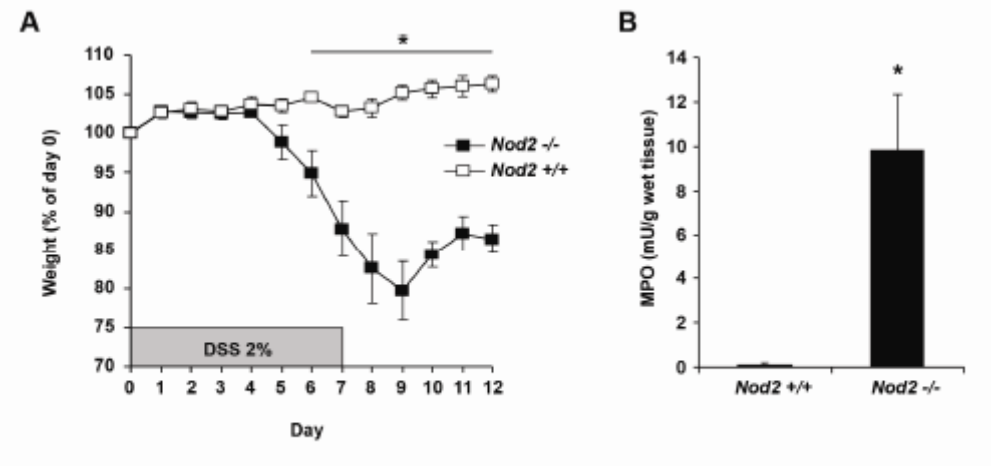


Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

4. CONCLUSIONS

These studies show that an increase in intestinal permeability (both in a transitory and a chronic form) leads to an increase in the mucosal resistance to inflammation through the induction of regulatory T cells. Therefore, an increase in the intestinal permeability with a consequent increase in the exposure of the host to products of the intestinal microbiota could represent an important mechanism of induction and maintenance of the intestinal tolerance homeostasis. The observation that mice with a *Nod2* gene deletion have an increase in the CD4 + LAP + regulatory cells, which determines a slighter damage following the induction of colitis, clearly shows that this genetic alteration by itself is not sufficient for the onset of Crohn's disease. The greater regulatory response to the microflora associated with the *Nod2* mutation could explain why the lack of Nod2 is not sufficient to establish inflammatory lesions in the experiment animal and to provide an interpretation for the established presence of an increased intestinal permeability in healthy relatives of patients suffering from Crohn's disease, notwithstanding the presence of the same genetic polymorphisms.

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