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Absence of NOD2 receptor predisposes to intestinal inflammation by a deregulation in the immune response in hosts that are unable to control gut dysbiosis

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ABSTRACT

Mutations in NOD2 predisposes to Inflammatory Bowel Diseases. Therefore, we evaluated the role of this innate receptor in the modulation of immunity in face of host microbiota changes. NOD2$^{-/-}$ mice presented higher susceptibility to experimental colitis than WT, with increased CD4 and CD8 T lymphocytes in the spleen. NOD2 deficiency also led to reduced Th17-related cytokines in the colon, with overall augmented IFN-γ in the gut and spleen. Nonetheless, there was increased frequency of CD4^{+}IL-4^{+} cells in the mesenteric lymph nodes besides elevated CTLA-4 and FoxP3 regulatory markers in the spleen of NOD2$^{-/-}$ mice, although it did not result in more efficient control of gut inflammation. Indeed, these animals also had augmented IL-1β and IL-5 in the peritoneum, indicating that this receptor may be important to control bacteria translocation too. Microbiota exchanging between cohoused WT and NOD2$^{-/-}$ mice led to colitis worsening in the absence of the receptor, while antibiotic therapy in WT mice abrogated this effect. Then, not only the genetic mutation confers increased susceptibility to inflammation, but it is also influenced by the microbiota harbored by the host. Finally, NOD2$^{-/-}$ mice are more prone to intestinal inflammation due to deregulated immune response and increased susceptibility to colitogenic bacteria.

1. Introduction

Nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) are a family of pattern recognition receptors which behave as intracellular sensors recognizing cytosolic pathogens, microbial products and other danger signs (Inohara and Nunez, 2003). The NLRs are crucial in regulating the innate immune response and thus play an important role in defense against intracellular pathogens (Ogura et al., 2001).

NOD2 belongs to the NLRs family and is expressed by monocytes, macrophages, dendritic cells and Paneth cells in the gut (Gutierrez et al., 2002). Its activation occurs by the recognition of muramyl dipeptide (MDP), a peptidoglycan present in gram-positive and gram-negative bacteria. The signaling cascade promoted by NOD2-MDP leads to recruitment of a protein kinase RICK (serine threonine kinase), also known as RIP2 (receptor-interacting protein 2) that is essential for the activation of NF-kB and MAPK, resulting in secretion of pro-inflammatory cytokines and chemokines like IL-1β, IL-6, CXCL8/IL-8, CXCL1/KC, CXCL2, CCL2 and RANTES (Franchi et al., 2009).

Genetic mutation in NOD2 is associated with susceptibility to Inflammatory Bowel Diseases (IBD), especially Crohn's disease (CD) (Hugot et al., 2001), in which the gut microbiota is recognized by NLRs, triggering the inflammatory process in the mucosa (Inohara and Nunez, 2003). Although there have been described several polymorphisms in IBD such as OCTN1/2 gene (organic cation transporter), NLRP3 gene (NOD like receptors family, pyrin domain containing 3) and TNFSF15

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gene (encoding TLA1 - a member of the superfamily TNF) among others (Ishihara et al., 2009), it is largely known that patients with mutations in NOD2 are highly susceptible to disease development and the absence of this receptor leads to an imbalance of the immune response that predispose to intestinal inflammation (Jiang et al., 2013). However, the mechanisms involved in NOD2 dysfunction and development of IBD associated with breakdown of immune tolerance in face of the intestinal microbiota are still controversial.

In this context, here we showed that besides the modulation of immune responses after barrier disruption, NOD2 protects against colitis by shaping the local microbiota that triggers the uncontrolled intestinal inflammation. Therefore, the absence of a functional receptor not only facilitates the immune deregulation but also prompted the host to intestinal inflammation due to increased susceptibility to colitogenic bacteria.

2. Material and methods

2.1. Animal studies

Male C57BL/6 wild type - WT or NOD2$^{-/-}$ mice, aged 6–8 weeks, weight 20–25 g, were housed under controlled temperature (25 °C), in specific pathogen-free and standard controlled environmental conditions, with a 12 h light/dark cycle. Mice were maintained isolated in cages with filtered air, sterile water and food provided ad libitum. Any mice handling during the experimental period (such as weight and scores evaluation) was performed inside a laminar flow cabinet. Animals were divided in control or colitis WT and NOD2$^{-/-}$ groups that were kept in single housing (SH) or cohousing (CH) conditions. Then, the groups studied were as follows. WT SH: wild type C57BL/6 mice in single housing conditions, exposed to dextran sulfate of sodium. WT SH (+ ABX): wild type C57BL/6 mice in single housing conditions, treated with antibiotics and exposed to dextran sulfate of sodium. WT CH: wild type C57BL/6 mice in cohousing conditions with NOD2 deficient mice, exposed to dextran sulfate of sodium. WT CH (+ ABX): wild type C57BL/6 mice treated with antibiotics, placed in cohousing conditions with NOD2 deficient mice and exposed to dextran sulfate of sodium. NOD2$^{-/-}$ SH: NOD2 deficient mice in single housing conditions, exposed to dextran sulfate of sodium. NOD2$^{-/-}$ CH: NOD2 deficient mice in cohousing conditions with WT, exposed to dextran sulfate of sodium. NOD2$^{-/-}$ CH (+ ABX WT): NOD2 deficient mice exposed to dextran sulfate of sodium in cohousing conditions with WT animals that were previously treated with antibiotics. Moreover, in every single round of colitis induction (in SH, CH or antibiotic-therapy conditions), a calibrator group of WT SH mice was exposed to DSS, simultaneously to the others, to provide an experiment calibration and avoid putative interferences related to colitis variation caused by any environmental conditions, throughout all experimental procedures.

All studies were performed in accordance with the Institutional Animal Care and Use Committee at Federal University of Triângulo Mineiro, Uberaba, Minas Gerais, Brazil. All procedures were approved under protocol 93. The experiments, which had 5 mice per group, were repeated three times to check the reproducibility of results.

2.2. DSS-induced colitis and clinical assessment of disease

Colitis was induced by 3% dextran sodium sulfate (DSS - MP Biomedicals, Illkirch, France. Molecular weight: 36,000–50,000 kDa) added to the drinking water during 9 days. Besides water and food intake, in all experiments the animals were evaluated daily for body weight and clinical signs of disease to summarize a clinical disease score, as previously described (Sales-Campos et al., 2015). In brief, the parameters evaluated were weight loss, wet anus, diarrhea, bleeding stools, hypoactivity and piloerection. Each sign presented by the mouse was scored as one point (1.0). The loss of weight between 5% and 10% from one day to another scored one point (1.0) too, while mice that lost more than 10% within the same period received two (2.0) points. The daily scores were obtained by the sum of all signs presented by the animal, as described above. The “overall score” represented the overall clinical condition of colitis mice, obtained by the sum of all daily clinical scores of each mouse, throughout the experimental period.

2.3. Euthanasia and collection of samples

Mice were euthanized on the 9th day of colitis and had the colon, spleen and mesenteric lymph nodes collected for further analysis. The colon samples were divided into smaller fragments and one of these fragments was immersed in PBS/10% formaldehyde for paraffin embedding and histopathological analysis. The other fragments were frozen immediately in liquid nitrogen for cytokine quantification or immersed in culture medium RPMI 1640 containing 5% fetal bovine serum (FBS) for flow cytometry experiments. The spleen and mesenteric lymph nodes were similarly immersed in RPMI 1640 5% FBS for flow cytometry assays.

2.4. Histopathological analysis

The large intestine samples were fixed in PBS/10% formaldehyde followed by standard histology procedures and paraffin embedding. Microtomy was performed to obtain 5 μm (μm) thick sections that were later stained by hematoxylin and eosin (H&E) for microscopic analysis of tissue inflammation. The mainly signs of microscopic intestinal inflammation evaluated were mucosal architecture and thickness, cellularity of the lamina propria, epithelial abnormalities and ulceration. The criteria for diagnosing Inflammatory Bowel Disease were adapted from a previous report from the British Society of Gastroenterology (Jenkins et al., 1997). To analyze mucosal thickness, lamina propria cellularity and colonic crypts, images of hematoxilin-eosin stained slides were acquired by a digital video camera (Evolution MP 5.0 – color – Media Cibernetic) connected to a light microscope (Nikon – Eclipse 50i), using the 20X and 40X objectives. The “ImageJ” software (http://rsb.info.nih.gov/ij/) was used for morphometric analysis. To determine mucosal thickness, we performed 30 measures from the muscularis mucosa to the superficial epithelia for each sample (mouse), in all groups. To calculate the density of cells per mm^2 of lamina propria, tissue infiltrate was counted in 15 different pre-determined areas of 375,832 μm^2 (each one), which involved normal or inflamed gut, per animal. The colon crypts were quantified per linear millimeter of muscularis mucosa.

2.5. Isolation of leukocytes from spleen, lymph nodes, intraepithelial compartment and lamina propria

Cells from spleen and mesenteric lymph nodes (MLNs) were obtained and filtered through a 70 μm cell strainer (BD Biosciences, Heidelberg, Germany). Red blood cells were lysed using ACK (Ammonium-Chloride-Potassium) buffer. Colon leukocytes (intraepithelial compartment leukocytes - IEL and lamina propria – LP) were isolated according to previous studies (Arstila et al., 2000; Cardoso et al., 2008). All cell suspensions were tested for viability by Trypan blue dye at 0.2% in a Neubauer chamber before being used in the culture and/or immunophenotyping experiments.

2.6. Flow cytometry

For phenotypic characterization of the leukocytes from spleen, MLN, LP or IEL, the following monoclonal antibodies conjugated to fluorophores were used: CD11b PE, CD11c FITC, CD3 PE, CD4 PECy5, CD8 FITC and γδ FITC (BD Pharmingen™, San Diego, USA). In brief, cells were incubated with 100 μl of PBS containing denaturized milk at 1% during 20 min at room temperature, followed by addition of surface staining antibodies for 20 min at room temperature in the dark. The