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Joint NOD2/RIPK2 Signaling Regulates IL-17 Axis and Contributes to the Development of Experimental Arthritis

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Intracellular pattern recognition receptors such as the nucleotide-binding oligomerization domain (NOD)-like receptors family members are key for innate immune recognition of microbial infection and may play important roles in the development of inflammatory diseases, including rheumatic diseases. In this study, we evaluated the role of NOD1 and NOD2 on development of experimental arthritis. Ag-induced arthritis was generated in wild-type, *NOD1*$^{-/-}$, *NOD2*$^{-/-}$, or *receptor-interacting serine-threonine kinase 2*$^{-/-}$ (*RIPK2*$^{-/-}$) immunized mice challenged intra-articularly with methylated BSA. Nociception was determined by electronic Von Frey test. Neutrophil recruitment and histopathological analysis of proteoglycan lost was evaluated in inflamed joints. Joint levels of inflammatory cytokine/chemokine were measured by ELISA. Cytokine (IL-6 and IL-23) and NOD2 expressions were determined in mice synovial tissue by RT-PCR. The *NOD2*$^{-/-}$ and *RIPK2*$^{-/-}$, but not *NOD1*$^{-/-}$, mice are protected from Ag-induced arthritis, which was characterized by a reduction in neutrophil recruitment, nociception, and cartilage degradation. NOD2/RIPK2 signaling impairment was associated with a reduction in proinflammatory cytokines and chemokines (TNF, IL-1β, and CXCL1/KC). IL-17 and IL-17 triggering cytokines (IL-6 and IL-23) were also reduced in the joint, but there is no difference in the percentage of CD4$^+$ IL-17$^+$ cells in the lymph node between arthritic wild-type and *NOD2*$^{-/-}$ mice. Altogether, these findings point to a pivotal role of the NOD2/RIPK2 signaling in the onset of experimental arthritis by triggering an IL-17–dependent joint immune response. Therefore, we could propose that NOD2 signaling is a target for the development of new therapies for the control of rheumatoid arthritis. *The Journal of Immunology*, **2012, 188: 5116–5122.**

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a family of intracellular microbial sensors that are proposed to survey the cytoplasm for the presence of microbial invaders and endogenous danger signals. It is currently widely accepted that NLR proteins are critical for the regulation of the innate immune response and, therefore, play a critical role in host defense against intracellular pathogens (1, 2).

NOD1 and NOD2 were the first members of the family to be described and are the most well studied (1, 2). Although NOD1 is ubiquitously expressed, NOD2 expression is restricted to monocytes, macrophages, dendritic cells, and intestinal Paneth cells (3). Initial biochemical characterization of NOD1 and NOD2 revealed that both induce NF-κB activation in a TLR-independent fashion (1). Subsequent analysis demonstrated that NOD1 and NOD2 recognize different structural core motifs derived from bacterial peptidoglycan (PGN). NOD1 activity is triggered by meso-diaminopimelic acid, which is unique to PGN structures from all Gram-negative bacteria and certain Gram-positive bacteria, including *Listeria* and *Bacillus* (4). In contrast, NOD2 is activated by muramyl dipeptide (MDP), a PGN motif present in all Gram-positive and Gram-negative bacteria (4). Upon ligand recognition, NOD1 and NOD2 undergo conformational changes and self-oligomerization. This is followed by the recruitment and activation of receptor-interacting serine-threonine kinase 2 (RIPK2; RICK/RIP2), which is essential for the activation of NF-κB and MAPKs (5, 6).

Despite their undisputed importance in host defense, definitive biological roles for most NLRs are as yet unknown. A recent study showed that NOD1 and NOD2 are expressed in the synovium of rheumatoid arthritis (RA) patients, and activation of these receptors leads to the production of proinflammatory mediators by synovial cells (7, 8). This evidence suggests that the activation of NLRs expressed in joint tissue might contribute to the initiation, amplification, and even to the progression of the inflammatory response in RA by sensing products of pathogens or damaged cells

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Abbreviations used in this article: AIA, Ag-induced arthritis; DMMB, 1,9-dimethylmethylene blue; i.a., intra-articular; mBSA, methylated BSA; MDP, muramyl dipeptide; NLR, nucleotide-binding oligomerization domain-like receptor; NOD, nucleotide-binding oligomerization domain; PGN, peptidoglycan; RA, rheumatoid arthritis; RIPK2, receptor-interacting serine-threonine kinase 2; WT, wild-type.

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or endogenous substances found in arthritic joints. In this context, it was recently demonstrated that joint inflammation induced by bacterial products is reduced in genetically deficient NOD2 mice (7). In this study, we investigated the involvement and the role of NLR (NOD1 and NOD2) in the genesis of arthritis using an experimental model of this disease (Ag-induced arthritis [AIA] in mice). Our results show that NOD2, but not NOD1, regulates the induction of arthritis in mice. Moreover, NOD2 signaling triggers the inflammatory immune response into the joint by a mechanism that seems to be dependent on the activation of RIPK2.

Materials and Methods

Animals

Wild-type (WT) C57BL/6-, *NOD1*-, *NOD2*-, and *RIPK2*-deficient ($^{-/-}$) (9–13) mice were used in this study. All knockout mice were generous gifts from Dr. Richard A. Flavell (Yale University School of Medicine) and were maintained in the Faculty of Medicine of Ribeirão Preto (University of Sao Paulo, Sao Paulo, Brazil). The genetic status of deficient mice was checked by PCR before the experiments (data not shown). All experiments were conducted according to the guidelines of the Ethics Committee of the School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil.

Induction of experimental arthritis and neutrophil migration evaluation

C57BL/6 and knockout mice were sensitized as previously described (14). Arthritis was induced in the immunized animals 21 d after the initial injection by intra-articular injection of methylated BSA (mBSA) dissolved in 10 μl PBS. Sham-immunized and immunized mice were challenged with mBSA or with PBS. Mice were killed 24 h after intra-articular injections, and neutrophil migration was determined as previously described (15) for total and differential cell counts. Results were expressed as the number of neutrophils per cavity. For evaluation of cartilage damage, mice were challenge intra-articularly twice with an interval of 7 d between the challenge and samples collection.

Mechanical hypernociceptive test

The term hypernociception rather than hyperalgesia or allodynia is used to define the decrease of the nociceptive withdrawal threshold in animal models. Mechanical articular hypernociception was evaluated as previously described by Pinto et al. (16).

Proteoglycan quantification assay

Proteoglycan contents were determined using the method described by Burkhardt et al. (14) with modifications. In brief, patella were carefully collected from each animal and fixed with formaldehyde (4%) overnight using a shaker. They were then transferred into a solution of formic acid (5%) and incubated for 4 h using a shaker for decalcification. Each patella was then placed into 100 μl papain digestion buffer consisting of a papain suspension (5 mg/ml) in calcium and magnesium-free PBS with 5 mM cysteine and 10 mM EDTA, pH 7.4. The samples were sealed and incubated in a humidified container in a 60°C oven for 16 h. After reaching room temperature, the samples were centrifuged for 10 min at 1000 × *g* to collect the condensation droplets. Next, 50 μl of the supernatants and of serial chondroitin sulfate solutions (standard curve; 50–1000 μg/ml) was placed into 96-well microtiter plates. The chondroitin sulfate STD solutions were also incubated with papain digestion buffer. Then, 300 μl of a 1,9-dimethylmethylene blue (DMMB; 50 mg/l) solution was added to each well, and the absorbance at 525 nm was measured immediately in a plate reader. The GAG content was calculated from the standard curve. The DMMB solution was prepared by dissolving 50 mg DMMB in 5 ml ethanol and diluting to a volume of 1000 ml with 0.2% (w/v) sodium formate buffer, pH 3.5. All reagents were purchased from Sigma-Aldrich.

Histological analysis

Whole knee joints were removed and fixed in 4% formaldehyde for 5 d before decalcification in 5% formic acid and processing for paraffin embedding. Tissue sections (7 μm) were stained with safranin O (cartilage PG loss). PG depletion was scored in the safranin O-stained slides on a scale from 0 to 100, ranging from stained cartilage to fully destained cartilage. All reagents were purchased from Sigma-Aldrich. For the evaluation of histological slides, we used ImageJ. The images were captured onto the hard drive of the workstation computer. Thereafter, captured images were opened in NIH Image/ImageJ for evaluating indices of positivity on histological slides. We used the "area method": the total area occupied by positive (red) label was estimated by setting a "threshold" using ImageJ's thresholding tool for selection of these positive areas separately. From this positive area data, the positive histological index for that image was calculated. Moreover, we used the "ROI Manager" (Region of Interest Manager) where we specify and draw out areas to be evaluated, editing out the unwanted elements. The results are demonstrated in percentage relative to total area of cartilage.

Real-time PCR quantitation

Three hours after mBSA injection, mice were terminally anesthetized and the synovial membrane was collected. Total RNA extraction, reverse transcription, and quantitative real-time RT-PCR were performed as previously described (17). Data were analyzed with the comparative cycle threshold method. Primer pairs for mouse β-actin, NOD2, IL-6, and IL-23 were as follows: *β-actin*, forward 5′-AGC TGC GTT TTA CAC CCT TT-3′ and reverse 5′-AAG CCA TGC CAA TGT TGT CT-3′; *NOD2*, forward 5′-CTT CAT TTG GCT CAT CCG TAG-3′ and reverse 5′-CTG GAG ATG TTG CAG TAC AAA G-3′; *IL-6*, forward 5′-AAC GAT GAT GCA CTT GCA GA-3′ and reverse 5′-GAG CAT TGG AAA TTG GGG TA-3′; *IL-23 p19*, forward 5′-AAC AGA TGC CCA GCC TGA CTT CTA-3′ and reverse 5′-AGG CCA ACC GCT CGA GAC TTT ATT-3′.

ELISA

Levels of IL-2, IL-17, TNF-α, IL-1β, and CXCL1/KC at 3 and 12 h after injection of mBSA were quantified in the joint tissues of immunized or nonimmunized mice and PBS-injected immunized mice by ELISA. In brief, joints were dissected out, frozen with liquid nitrogen, crushed in a mortar and pestle, then solubilized in PBS and measured using ELISA (15), with the results expressed as picograms per milliliter.

Proliferation assay

In brief, lymph node cells from WT or knockout mice cultured at 5×10^5 cells/well were incubated with the indicated stimuli Con A (0.5 μg/ml) or mBSA (100 μg/ml) for 4 d in round-bottom, 96-well plates in 200 μl RPMI 1640 complete medium. [^{3}H]Thymidine (1 μCi/well; PerkinElmer, Boston, MA) was added for the last 24 h of incubation. Each condition was studied in triplicate. Cells were collected on filter paper (Wallac, Turku, Finland) using a cell harvester (Perkin Elmer), and [^{3}H]thymidine incorporation was measured with a beta counter (Molecular Devices).

Characterization of intracellular cytokine profile of CD4$^+$ T cells in the lymph nodes of arthritic mice: staining and flow cytometry

At 14 d after immunization, cells from lymph node of naive or immunized mice were harvested. For immunostaining of intracellular cytokines, cells were stimulated and permeabilized before cell staining. In brief, cells were suspended in FACS lysing solution (Becton Dickinson) at room temperature for 10 min and then stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) during 4 h in culture medium containing 2 μM brefeldin A (Golgi Stop; Becton Dickinson). Cells were washed once with PBS and stained for surface markers (anti-CD4 FITC) and intracellular markers (anti–IFN-γ allophycocyanin and anti–IL-17 PE) using Cytofix/Cytoperm kit, according to manufacturer's instructions (Becton Dickinson). After staining, cells were acquired in a flow cytometry (FACSCanto II; Becton Dickinson) and analyzed using FlowJo software (version 7.6.1; TreeStar Software). Doublets were excluded from analyses by forward scatter area and forward scatter height, lymphocytes were gated by the region determined by forward scatter and side scatter; then CD4$^+$ cells were gated by FITC intensity. In the gated CD4$^+$ mononuclear cells, IL-17 PE and IFN-γ allophycocyanin-positive cells were determined.

Anti-mBSA Ab titer determination

Serum anti-mBSA Ab titers in pooled sera from WT, NOD1$^{-/-}$, and NOD2$^{-/-}$ mice were measured by ELISA. In brief, 96-well plates were coated with 50 μl mBSA solution (10 μg/ml, in 0.1 M phosphate buffer) overnight at 4°C. Thereafter, serial dilutions of sera were added and incubated overnight at 4°C. Bound, total IgG was detected with biotin-conjugated anti-mouse IgG (Vector). Finally, 50 μl avidin-HRP (1:5000 dilution; DAKO, Glostrup, Denmark) was added to each well, and after 30 min, the plates were washed and the color reagent OPD (200 μg/well; Sigma) was added. After 15 min, the reaction was stopped with 1 M H_2SO_4 and the OD read at 490 nm.

Statistical analysis

The data are reported as means ± SEM and are representative of two or three independent experiments. The means from different treatments were compared by ANOVA. When statistical significances were identified, individual comparisons were subsequently tested with Bonferroni's *t* test for unpaired values. Statistical significance was set at $p < 0.05$.

Results

NOD2, but not NOD1, is involved in the pathogenesis of experimental arthritis

To explore the role of NOD1 and NOD2 in the pathogenesis of arthritis, we used the model of AIA with mBSA as Ag. In this study, it was observed that 24 h after mBSA challenge in the knee joints of WT and *NOD1*$^{-/-}$ immunized mice, there was a prominent joint neutrophil infiltration and mechanical hypernociception. These inflammatory events were reduced in *NOD2*$^{-/-}$ immunized mice (Fig. 1A, 1B). In an attempt to evaluate the cartilage erosion in mBSA-induced arthritis, we quantified the levels of proteoglycan in the joints of mice 7 d after a second challenge with mBSA. In this set of experiments, we observed that WT and *NOD1*$^{-/-}$ mice exhibited significantly lower proteoglycan levels compared with control mice, whereas this proteoglycan reduction was not observed in *NOD2*$^{-/-}$ mice (Fig. 1C). These results can be directly visualized by histopathological analysis in safranin-stained cartilage slices (Fig. 1D).

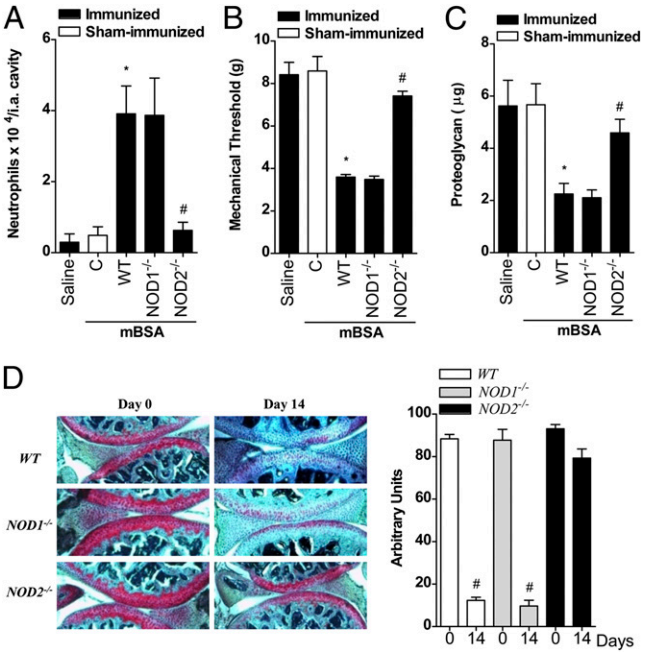

FIGURE 1. NOD2, but not NOD1, is involved in AIA. (**A**) WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ immunized and sham-immunized mice were challenged with mBSA (10 μg; i.a.) or saline, and 24 h later, neutrophil migration was determined in synovial fluid. (**B**) The nociceptive threshold was evaluated 7 h after i.a. injection of mBSA (30 μg; i.a.) or saline in WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ immunized or sham-immunized mice. (**C**) Evaluation of proteoglycan loss induced by i.a. injection of mBSA (30 μg; i.a.) or saline in WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ immunized or sham-immunized mice. Saline-challenged immunized mice were used as a control. (**D**) Histological analysis of articular cartilage in WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ immunized mice after mBSA (30 μg; i.a.) challenge. Knee joints were stained with safranin O-fast green. Three independent experiments were performed, and results are expressed as means ± SEM of five mice. Original magnification ×40. $^{*}p < 0.05$ versus sham-immunized mice, $^{\#}p < 0.05$ versus WT mice after mBSA challenge.

NOD2 modulates proinflammatory cytokines release and expression in the joints

The decrease in the joint inflammatory parameters in *NOD2*$^{-/-}$ mice was associated with a reduction in the levels of proinflammatory cytokines and chemokines, including TNF-α, IL-1β, and CXCL1/KC (Fig. 2A–C). We also observed that synovial membrane of WT arthritic mice presents an elevated mRNA expression of NOD2 3 h after mBSA challenge (Fig. 2E). To confirm these data, we analyzed the levels of NOD2 protein in synovial tissue using immunofluorescence. We observed an increased expression of NOD2 receptor in synovial tissue of arthritic mice versus sham-immunized WT mice, as well as in NOD1$^{-/-}$ mice. As expected, the expression of NOD2 was not found in NOD2$^{-/-}$ challenged mice (Supplemental Fig. 1).

Impairment of *NOD2* was also associated with a reduction in IL-17 expression (Fig. 2D) and with a reduction in IL-17 triggering cytokines (mRNA for IL-6 and IL-23) in the synovial tissue (Fig. 2F, 2G). In contrast, the production and mRNA expression of these cytokines in *NOD1*$^{-/-}$ mice was not different from WT mice (Fig. 2A–D, 2F, 2G).

NOD2-deficient mouse-derived lymph node cells exhibit normal proliferative response

To investigate whether the reduced response of *NOD2*$^{-/-}$ mice was due to a reduced immunization state, we determined the levels of Ab against mBSA in the serum of immunized mice; proliferative response and IL-2 levels in the supernatant of lymph node-derived cells stimulated with mBSA or with nonspecific stimulus (Con A) were also evaluated. It was observed that proliferative response and IL-2 production in lymph node cultures of previously immunized mice stimulated with mBSA or with nonspecific Con A and the serum levels of Ab against mBSA were similar in WT and *NOD2*$^{-/-}$ mice (Fig. 3A–C). These experiments suggested that *NOD2*$^{-/-}$ mice were immunized to the same extent as WT mice.

We also characterized T cell phenotype in the lymph node of arthritic mice. There is no difference in the percentage of CD4^{+} IL-17^{+} or CD4^{+} IFN-γ^{+} cells between WT and *NOD2*$^{-/-}$ at 14 d after immunization (Fig. 3D, 3E).

The NOD2 effector RIPK2 participates in AIA

In the next step, we analyzed whether the most important NOD2 downstream signaling molecule, RIPK2, also participates in AIA. The intra-articular (i.a.) injection of mBSA in WT immunized mice induced neutrophil migration and mechanical hypernociception that was reduced in *RIPK2*$^{-/-}$ immunized mice (Fig. 4A, 4B). Furthermore, the mBSA challenge did not promote cartilage erosion in *RIPK2*$^{-/-}$ mice that was observed in WT mice (Fig. 4C). The levels of IL-17, TNF-α, and KC/CXCL1 chemokine, but not IL-1β, were reduced in *RIPK2*$^{-/-}$ mice induced by mBSA in immunized mice (Fig. 4D–G).

Discussion

The NLRs play key roles in the initiation of innate immune response to pathogen-derived molecules or endogenous metabolites of damaged cells (18). The presence of NOD1 and NOD2 in the synovial membrane of RA patients suggests that they could potentially be involved in the initiation, amplification, and progression of the inflammatory response in rheumatic diseases (8). In this study, this hypothesis was investigated in a mouse model of this disease. Indeed, our records show that NOD2 and its most important downstream signaling molecule, RIPK2, participate in the cascade of events involved in the genesis of AIA. It seems that NOD2/RIPK2 signaling is important to drive IL-17–triggering cytokines in the joint.

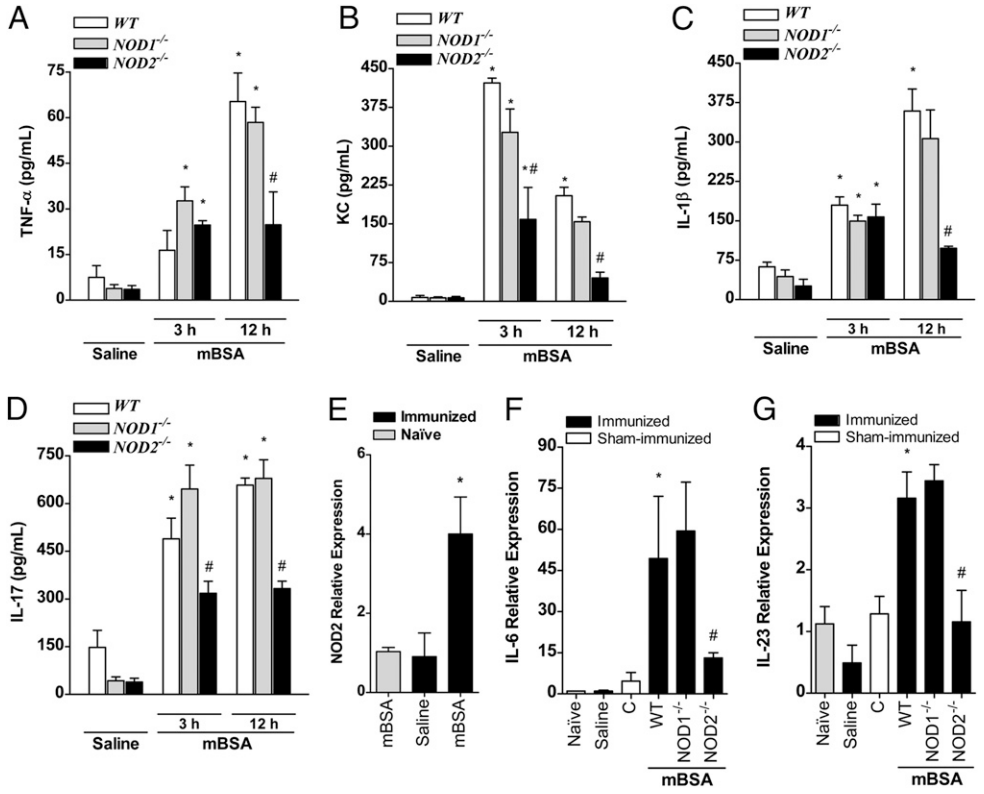

FIGURE 2. Role of NOD2 in triggering the local IL-17 immune response. The concentrations of TNF-α (**A**), KC (**B**), IL-1β (**C**), and IL-17 (**D**) in the articular cavity of WT, *NOD1*$^{-/-}$, or *NOD2*$^{-/-}$ mice were determined 3 and 12 h after challenge with mBSA (10 μg; i.a.) or saline in immunized mice or sham-immunized mice. mRNA expression of NOD2 (**E**), IL-6 (**F**), and IL-23 (**G**) in WT, *NOD1*$^{-/-}$, or *NOD2*$^{-/-}$ synovial tissue samples was determined by real-time RT-PCR relative to β-actin ($2^{-\Delta Ct} \times 1000$) expression. Synovial tissues were collected 3 h after i.a. injection of saline (control) or mBSA (10 μg; i.a.) in WT, *NOD1*$^{-/-}$, or *NOD2*$^{-/-}$ immunized or sham-immunized mice. Three independent experiments were performed, and results are expressed as means ± SEM of five mice per group. $^{*}p < 0.05$ versus control mice, $^{\#}p < 0.05$ compared with WT immunized mice after mBSA challenge.

Recent data have demonstrated that NOD2 is functional within the mouse joints (19). For instance, systemic or local (intra-articular) administration of MDP, an NOD2 agonist, PGN, or *Streptococcus pyogenes* cell wall fragments results in significant NOD2-dependent joint inflammation (7, 19–21). It is important to mention that these arthritic models are generated by administration of bacterial components and are triggered mainly by mechanisms related to innate immunity. To examine the involvement of NOD2 in adaptive immune inflammation, we used a model of arthritis induced by mBSA as an Ag in previously immunized mice. The mBSA-induced arthritis mouse model is a suitable and reproducible experimental system that exhibits several features with histopathological findings similar to those observed in human RA, including cartilage degradation (22). In this model, the adaptive immune response is dependent on CD4^{+} T cells and, to a lesser extent, is mediated by serum Ab (23). Therefore, we used this model to explore the role of NOD1 and NOD2 in the pathogenesis of experimental arthritis. Notably, our results showed an elevation of NOD2 mRNA expression on synovial membrane of mice challenged with mBSA-induced arthritis.

RA is a chronic inflammatory disorder characterized by leukocyte recruitment, pain, and progressive destruction of joints, resulting in disability (24). A previous report from our laboratory demonstrated the effector phase of AIA is characterized by an early massive infiltration of neutrophils in the joint cavity and also by an intense mechanical nociception, measured by a decrease in the mechanical nociceptive threshold (16). The inflammatory events in this model, including polymorphonuclear accumulation, are triggered by Ag-specific, CD4^{+}-derived mediators (23, 25). In addition to leukocyte recruitment and pain, the erosion of articular cartilage caused by extracellular matrix breakdown is also a hallmark of arthritis (26). In this study, prominent joint neutrophil infiltration, mechanical hypernociception, and cartilage erosion were not observed in *NOD2*$^{-/-}$ immunized mice. Moreover, decrease in the joint inflammatory parameters in *NOD2*$^{-/-}$ mice was associated with a reduction in the levels of proinflammatory cytokines. Regarding NOD1, differently from that has been demonstrated in other models of nonimmune arthritis (7); in AIA model, NOD1 did not present an anti-inflammatory role. The nature of the immune response might account for this discrepancy.

One intriguing finding of our study is that NOD2-deficient mouse-derived lymph node cells exhibit normal production of IL-2 and a normal proliferation response when stimulated by the specific Ag. Therefore, under these experimental conditions, the reduced inflammatory response in NOD2-deficient mice is not related to the immunization process, in which constituents of CFA could be activating NOD2. These results suggest that the role of NOD2 in arthritis might involve events triggered locally in the joints after mBSA challenge.

The inevitable question that emerges from these findings is: how does the NOD2 receptor modulate the joint inflammatory response? The inflammatory events of RA are primarily mediated by cytokines and chemokines. The current hypothesis is that IL-17 plays a pivotal role in the pathophysiology of RA (27). This hypothesis is supported by studies that have shown that pharmacological or genetic inhibition of IL-17 production or activity attenuates the events of experimental arthritis, such as leukocyte migration, osteoclast activation, and cartilage and bone erosion (28). Corrob-

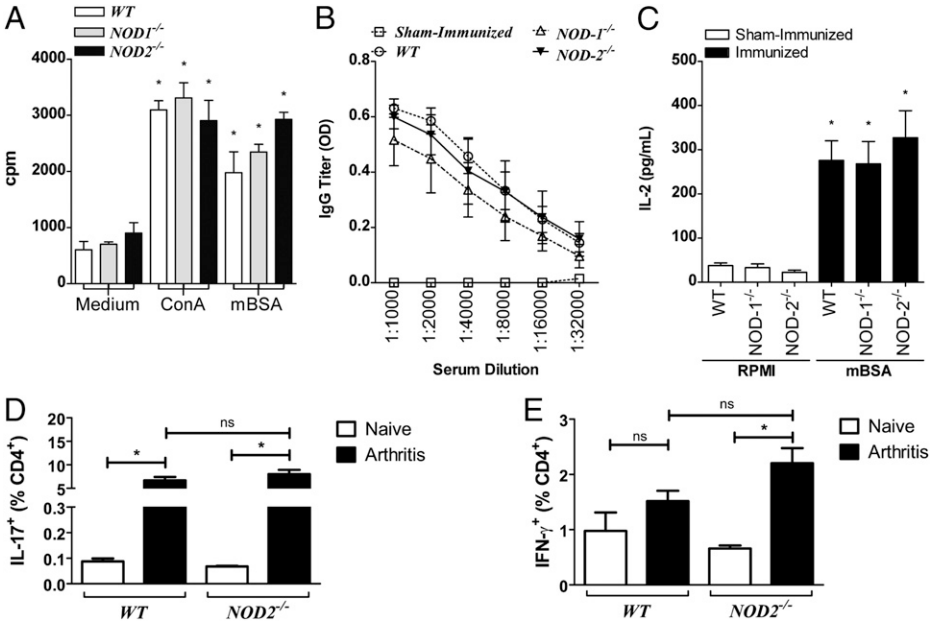

FIGURE 3. Immunization state of WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ mice. (**A**) Lymph node-derived cells (5×10^5/well) from WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ immunized mice were cultured in RPMI 1640 in the presence of Con A (0.5 μg/ml) or mBSA (100 μg/ml), and 96 h later, proliferation response was evaluated. (**B**) Levels of Ab against mBSA in the serum of WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ immunized and sham-immunized mice. (**C**) Lymph node-derived cells (5×10^5/well) from WT, *NOD1*$^{-/-}$, and *NOD2*$^{-/-}$ immunized mice were cultured and stimulated with RPMI 1640 or mBSA (100 μg/ml), and 72 h later the levels of IL-2 in the supernatant were determined by ELISA. The percentages of CD4$^+$ cells expressing IL-17 (**D**) and IFN-γ (**E**) were analyzed in lymph node-derived cells from WT and *NOD2*$^{-/-}$ naive and immunized mice. Lymph nodes were harvested 14 d after immunization. Two independent experiments were performed, and results are expressed as means ± SEM of five mice or cultured cells from five animals. *$p < 0.05$ compared with medium-stimulated cells or sham-immunized mice.

orating with previous data, we detected high levels of IL-17 in the joint cavities of WT arthritic mice. However, these cytokine levels were remarkably reduced in *NOD2*$^{-/-}$ mice, indicating the importance of this receptor on modulation of IL-17–releasing cells.

Data from the literature suggest that Th17 cells are important for joint inflammation and its differentiation is initiated in draining lymph nodes, and these cells are recruited to the inflamed focus. However, recent evidence suggests that IL-17–producing cells may be locally generated in the synovium in response to local cytokines produced by inflammatory macrophages (29). In agreement with this hypothesis and with our suggestion that NOD2 might play a role in the local government of IL-17–producing cells, no difference was found in the percentage of CD4$^+$ IL-17$^+$ or IFN-γ$^+$ cells in the lymph nodes of WT and NOD2 null mice. Furthermore, mRNA expression levels of crucial cytokines that instruct and drive T cells to produce IL-17, such as IL-6 and IL-23, and

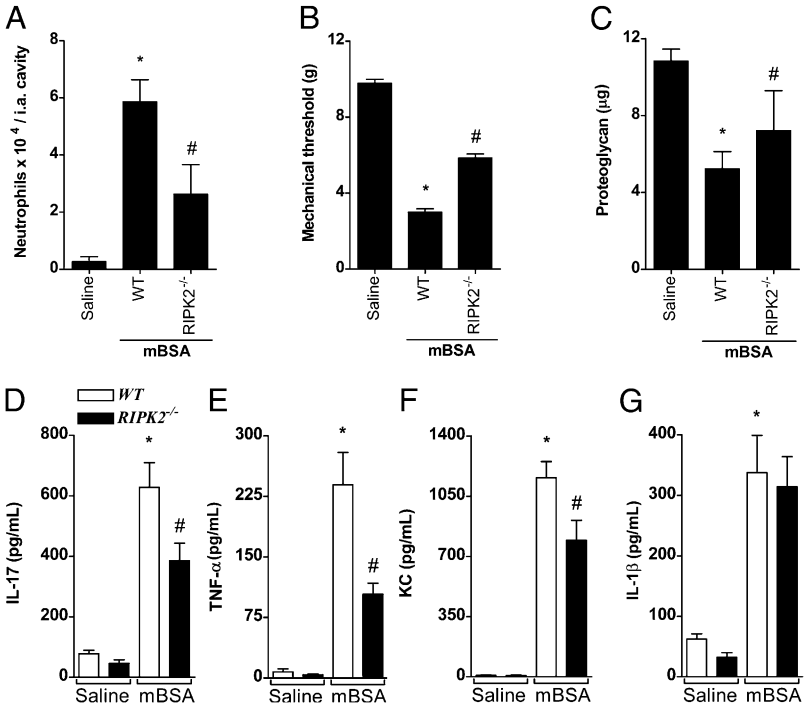

FIGURE 4. RIPK2 participates in AIA. (**A**) WT and *RIPK2*$^{-/-}$ immunized mice were challenged with mBSA (10 μg; i.a.) or saline, and 24 h later, neutrophil migration was determined in synovial fluid. (**B**) The nociceptive threshold was evaluated 7 h after i.a. injection of mBSA (30 μg; i.a.) or saline in WT or *RIPK2*$^{-/-}$ immunized mice. (**C**) Evaluation of proteoglycan loss induced by i.a. injection of mBSA (30 μg; i.a.) in WT and *RIPK2*$^{-/-}$ immunized mice. Saline-challenged immunized mice were used as a control. The concentrations of IL-17 (**D**), TNF-α (**E**), KC (**F**), and IL-1β (**G**) in the articular cavity were determined 12 h after challenge with mBSA or saline in immunized mice. Three independent experiments were performed, and results are expressed as means ± SEM of five mice per group. *$p < 0.05$ versus control mice, $^{\#}p < 0.05$ compared with WT immunized mice after mBSA challenge.

also the levels of IL-1β are strongly reduced in the synovium of NOD2 null mice. The importance of NOD2 in modulating these cytokines production is also supported by recent data showing that the activation of NOD2 in human dendritic cells leads to an enhancement of IL-17 production by memory T cells in an IL-23– and IL-1β–dependent manner (30). Despite our hypothesis that NOD2 drives IL-17 production by T cells in the joints, we could not discard the production of IL-17 by other synovial tissue cells after NOD2 activation. For instance, it was recently shown that only a small proportion of IL-17–expressing cells were T cells in human synovial tissue (31). Furthermore, the majority of IL-17 expression seems to be in mast cells (31).

RIPK2 is a ubiquitously expressed serine-threonine kinase originally associated with induction of apoptosis (32, 33). Interestingly, recent data demonstrated that RIPK2 is a key effector molecule downstream of NOD1 and NOD2, but not downstream of TLRs. Indeed, NOD signaling is impaired in macrophages from RIPK2-deficient mice (6). Furthermore, RIPK2 mice were protected against mBSA-induced arthritis and present reduction on articular joint levels of IL-17. Although the molecular mechanism by which activation of RIPK2 signaling stimulates the IL-17 immune response was not determined, there is evidence indicating that RIPK2 is the critical step between NOD2 and NF-κB activation, which may induce IL-17–promoting cytokines such as IL-23 and IL-6 (34). The levels of TNF-α and KC/CXCL1 chemokine in articular joint are managed by RIPK2. Notably, these last findings are also in line with the data showing that IL-17 is critical in stimulating the release of TNF-α and chemokines by joint structural and resident cells during arthritis (35).

The observations that the decreased inflammatory parameters such as neutrophil migration and proteoglycan degradation in RIPK2 null mice were less pronounced when compared with NOD2-deficient mice and that IL-1β production was not reduced in these null mice suggest that other signaling pathways might be acting downstream of NOD2. Although at this stage we did not identify the possible mechanism, there are two possibilities that we could suggest. First, NOD2 could be stimulating caspase recruitment domain family 9-dependent pathways (36). For instance, the activation of MAPKs by NOD2 is dependent on caspase recruitment domain family 9 that, in turn, stimulates AP-1 but not NF-κB–dependent gene expression (37). Second, NOD2 through its N-terminal caspase recruitment domain forms a complex with NLR family, pyrin domain containing 1 and caspase-1 that, in turn, activates caspase-1 to trigger IL-1β processing and secretion (38). It could explain our finding in which IL-1β production is reduced in NOD2 but not in RIPK2 null mice during AIA.

An important question that arose from our data is, how is the NOD2 signaling pathway activated in the context of human arthritic joints? Although future studies are necessary to answer this question, it was recently found that a high concentration of NOD2 ligand (MDP fragments) exists in the synovial tissue of RA patients (7). This hypothesis is in agreement with the idea that infections are implicated in the aggravation of RA. However, in our current experimental model (AIA), mBSA challenge into the joint is believed to produce a sterile immune response. In this context, it is important to note that NOD2 would also recognize products from damaged cells and endogenous metabolites as observed with other NLRs (18). In addition, to enhance local $CD4^+$ $Th17^+$ adaptive immune response as discussed earlier, it is likely that NOD2 activation in resident (synovial) or migrating (neutrophil) cells by endogenous or pathogen-derived agonists could amplify innate immune response in arthritic joints (39). Nevertheless, further investigations and manipulations of NOD2 signaling may improve therapeutic options for RA patients.

The major findings of this work underline the importance of NOD signaling in the cascade of events involved in the pathogenesis of arthritis. NOD2 seems to signal, at least in part, via RIPK2, which, in turn, mediates the development of arthritis. This pathway contributes to triggering the IL-17–dependent joint immune response by managing the local production of IL-6, IL-23, and IL-1β. Together, these events cooperate to promote joint inflammation. Our results suggest that NOD2 signaling constitutes a real research target for development of new therapeutics for the control of arthritis immune inflammation.

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Disclosures

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