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Nod2-deficiency augments Th17-responses and exacerbates autoimmune arthritis

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Abstract

Arthritis in genetically susceptible SKG-strain of mice models a theoretical paradigm wherein autoimmune arthritis arises due to interplay between preexisting autoreactive T cells and environmental stimuli. SKG mice have a point mutation in ZAP-70 that results in attenuated T cell receptor signaling, altered thymic selection, and spontaneous production of autoreactive T cells that cause arthritis following exposure to microbial β-glucans. Here we identify Nod2, an innate immune receptor, as a critical suppressor of arthritis in SKG mice. SKG mice deficient in Nod2 (Nod2$^{-/-}$SKG) developed a dramatically exacerbated form of arthritis, which was independent of sex and microbiota, but required the *skg*-mutation in T cells. Worsened arthritis in Nod2$^{-/-}$SKG mice was accompanied by expansion of Th17 cells, that to some measure co-produced TNF, GM-CSF and IL-22, along with elevated IL-17A levels within joint synovial fluid. Importantly, neutralization of IL-17A ameliorated arthritis in Nod2$^{-/-}$ SKG mice, indicating that Nod2-mediated protection occurs through suppression of the Th17 response. Nod2-deficiency did not alter Treg development or function. Instead, Nod2-deficiency resulted in an enhanced fundamental ability of SKG CD4^{+} T cells (from naïve mice) to produce increased levels of IL-17 and to passively transfer arthritis to lymphopenic recipients on a single-cell level. These data reveal a previously unconsidered role for T cell-intrinsic Nod2 as an endogenous negative regulator of Th17-responses and arthritogenic T cells. Based on our findings, future studies aimed at

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understanding a negative regulatory function of Nod2 within autoreactive T cells could provide novel therapeutic strategies for treatment of patients with arthritis.

Keywords

Rodents; Th17 cells; Autoimmunity; Arthritis; Transgenic/Knockout Mice

INTRODUCTION

Inflammatory arthritis is a chronic disorder of the joints involving inflammation of the synovial membrane, leukocyte recruitment, and progressive bone and cartilage destruction. Arthritis manifests in over 100 different rheumatic disorders including rheumatoid arthritis (RA), spondyloarthropathies (e.g. ankylosing spondylitis), psoriatic arthritis, systemic erythematosus lupus, and enteropathic arthritis (i.e. arthritis associated with inflammatory bowel disease). As a leading cause of disability in the United States, arthritis is a major public health concern due to its economic burden and impact on quality of life (1). Although the etiology of arthritis is poorly understood, a current paradigm proposes that complex interactions between genetic and environmental factors result in a break in immune tolerance and the generation and propagation of autoreactive T cells. Compelling data from clinical studies and experimental models of arthritis implicate a central role for $CD4^+$ T helper (Th) cells in the pathogenesis of disease. In particular, a lineage of $CD4^+$ T cells that produce the cytokine IL-17 (Th17 cells) is known to be pathogenic in arthritis (2, 3). Th17 cells promote the recruitment of neutrophils and activation of joint synoviocytes and chondrocytes, leading to inflammation and bone destruction (2, 3). Although Th17 immunity is understood to promote arthritis, anti-IL-17 biologics have had limited clinical benefit (4), thereby underscoring the complexity surrounding the role of Th17 cells in disease and that targeting IL-17 alone is not ideal. Thus, identification of additional mechanisms that engender a break in immune tolerance and control of autoreactive Th17 cells should be a critical focus of investigation.

Despite the wealth of knowledge about the role of the adaptive immune system in arthritis, its relationship with innate immunity is poorly defined. Intriguingly NOD2, a member of the Nod-like receptor (NLR) family, appears to play a role in arthritis, as a point mutation in this molecule leads to ~100% incidence of a heritable form of polyarticular arthritis as part of Blau Syndrome (5–7). Thus, studies investigating the biological function(s) of Nod2 could offer important insights into how a single gene might function in arthritis. Nod2, a pattern recognition receptor important for host defense against intracellular bacteria (8), was initially identified as being expressed predominantly by myeloid cells. However, *Nod2* expression has since been detected across hematopoietic and non-hematopoietic cells including T cells (9, 10), vascular endothelial cells (11), synoviocytes (12) and chondrocytes (13). Nod2 detects cytosolic bacteria by sensing peptidoglycan (PGN), of which muramyl dipeptide (MDP) is the minimal moiety required for its activation. Recognition of PGN by Nod2 activates RIP2, MAP kinase, and NF-κB as part of a signaling cascade that results in transcription of genes responsible for antimicrobial immunity (14, 15). More recently, Nod2 has been reported to participate in signaling responses outside of its “canonical” PGN-

sensing function, e.g. anti-viral responses, autophagy, and endoplasmic reticulum (ER) stress responses (16–19). Previous studies suggest a role for Nod2 in local, injurious responses of the synovium induced by intra-articular injection of PGN (20–23). However, our understanding of the role of Nod2 in the generation or function of autoreactive T cells remains limited.

Given the strong clinical link between NOD2 and rheumatic disease we sought to investigate the role of Nod2 in a T cell-mediated model of arthritis. SKG mice are genetically prone to arthritis due to an inherent mutation in the T cell signaling molecule, Zap-70 (24), which diminishes the strength of TCR signaling initiated by TCRξ and CD3 chains (25). Thus, in SKG mice, compromised central tolerance results in the escape of autoreactive T cells from the thymus into the periphery where they can become Th17 cells that target the joint (26). Additional signals, such as those provided by fungal-derived β-glucan polymers such as zymosan, are required for the subsequent generation of pathogenic Th17 cells and development of arthritis in SKG mice (27).

The studies here identify a previously unrecognized role for Nod2 as an essential protectant against development of arthritis. Absence of Nod2 (Nod2$^{-/-}$SKG mice) resulted in worsened form of arthritis, which was mediated by dysregulation of the Th17 response. An important finding from these studies is that Nod2-mediated protection was intrinsic to CD4^{+} T cells. In particular, this protection was not conferred through effects on Treg development or function, but rather on CD4^{+} T cell production of IL-17. Reconstitution of lymphopenic recipients with CD4^{+} T cells purified from naïve Nod2$^{-/-}$ SKG vs. SKG mice recapitulated the worsened phenotype observed in Nod2$^{-/-}$ SKG mice, thereby indicating a T cell-intrinsic function for Nod2 in control over arthritis. Future studies aimed at further understanding an endogenous negative regulatory function of Nod2 within autoreactive T cells could inform us of potentially novel therapeutic strategies for arthritis.

MATERIALS AND METHODS

Mice:

Nod2$^{-/-}$SKG mice were generated by breeding SKG mice (24) with Nod2$^{-/-}$ mice (Jackson Laboratory) that we had backcrossed 10 generations onto the BALB/c background. Nod2 deficiency, along with the G489T mutation in *Zap-70*, of SKG progeny were confirmed by PCR as described (28). Athymic *nu/nu* (nude) and Rag1$^{-/-}$ mice, both on BALB/c background (Jackson Laboratory), were bred in our facility. Nod2$^{-/-}$Rag1$^{-/-}$ mice were generated by breeding Nod2$^{-/-}$ mice with Rag1$^{-/-}$ mice, with the Nod2 deficiency confirmed by PCR. Animals were maintained under specific pathogen-free (SPF) conditions at the VA Portland Health Care System. All studies were conducted with 6 wk-old female mice, and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and institutional guidelines for animal welfare.

Induction and evaluation of arthritis:

Arthritis was induced by a single intraperitoneal (i.p.) injection of 1.5 mg zymosan (Invivogen) a cell wall-derived preparation of *Saccharomyces cerevisiae* enriched in β-

glucan polymers. Clinical arthritis for each paw was graded (0 – 4) in masked fashion using defined criteria (28) and scores for each paw were summed such that the total score per mouse ranged from 0 to 16. For calculation of disease incidence, a mouse was considered positive for arthritis when a total clinical score was greater than or equal to 1 and was maintained for 2 or more weeks. Bodyweight loss was calculated as percent change between bodyweights at the start and termination of study. For histological analysis of joints, dissected ankles were fixed, decalcified, and embedded in paraffin (28). Tissue sections (7 ¼m thick) were stained with hematoxylin and eosin (H&E) and examined using light microscopy (original magnification X100).

Histopathological analysis of enteritis:

Segments of the small intestine were coiled, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Sections (7 ¼m) were stained with H&E and twelve regions of the ileum of each mouse intestine were photographed (using 10X objective). To quantify the extent of disease, images were analyzed offline with ImageJ (NIH) by a pathologist masked to experimental condition. Data are expressed as percent lesion, defined as the percentage length of discrete inflammatory lesions (i.e. accumulations of mononuclear and polymorphonuclear inflammatory cells within the mucosa, submucosa and muscularis propria) relative to length of intestine within the photograph.

Near-infrared Imaging:

Upon termination of experiments, imaging was performed on legs and spines dissected from ProSense® 680-injected (NEV 10003, PerkinElmer) animals. Emitted intensities of the near-infrared signal, indicating levels of protease activity, were quantified within defined regions of interest (ROI), as previously detailed in the SKG model of arthritis (28). Signal intensities in ROI were first normalized to those of corresponding ROI from healthy, naïve, WT-BALB/c mice using the small animal image analysis application of Image Studio Software (LI-COR Biosciences) before quantification.

Co-housing/fecal transmission study:

SKG and Nod2$^{-/-}$SKG mice were housed in a 1:1 ratio to equally expose each genotype to a composite of fecal and bedding material, a strategy based on a prior recolonization study in SKG mice (29). Mice were cohoused starting at the time of weaning (3 wk of age) and throughout the duration of the study (i.e out to 8 wk post-zymosan).

Anti-IL-17 blockade:

For *in vivo* IL-17A neutralization studies, mice were i.p. injected with 100 ¼g of either anti-IL-17A mAb (Clone 50104; R&D Systems) or rat IgG2a isotype control (Clone 54447; R&D) on the day prior to zymosan challenge and weekly thereafter. This dosing regimen was based on a prior report of *in vivo* efficacy of IL-17 blockade in SKG mice (30).

ELISAs:

Cytokine levels for IL-17A/F, IL-10 and IFNγ were quantified by ELISA according to manufacturer's instructions (DuoSet® ELISA kits, R&D Systems). To measure IL-17A

levels in the intra-articular space of the joint, synovial fluid was aspirated from the synovial capsule of ankle joints using a 23G needle and immediately frozen at −20ºC. Equal volumes (1 ¼l) of synovial fluid were used to measure IL-17A by ELISA.

Flow cytometry:

Synovial fluid was aspirated from the sub-capsular space of ankle joints. Cells from ankle-draining (popliteal) lymph nodes and spleens were prepared by collagenase-digestion (*Clostridium histolyticum* Collagenase D, Roche) and filtration through a 70-¼m cell strainer. Erythrocytes were lysed using red blood cell lysis buffer (Sigma). Collected cells were blocked with mAb to FcγRIII/I (2.4G2, BD Biosciences) and subsequently incubated with mAb to the following surface molecules for T cells; Thy1.2 (53–2.1, BioLegend), CD8α (53–6.7, eBiosciences) and CD4 (RM4–5, BD Biosciences) along with LIVE/DEAD® Aqua stain (Life Technologies), then fixed in 4% paraformaldehyde (Sigma). For *in vitro* stimulation and intracellular cytokine staining, single cell suspensions from popliteal lymph nodes were prepared as above and seeded at 5 $\times 10^6$ cells/ml in 96-well plates in RPMI supplemented with 10% fetal bovine serum (endotoxin-free, Gemini Bio-Products). Cells were treated with 20 ng/ml PMA (LC Laboratories) and 1 ¼g/ml ionomycin (LKT Laboratories) vs. media for 5 h in the presence of Brefeldin A (1 ¼g/ml; BD Biosciences). Cells were blocked, stained for cell surface markers as above, fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences), after which they were stained with mAbs to IL-17A (TC11–18H10), TNFα (MP6-XT22), IFNγ (XMG1.2), IL-22 (IL22JOP), and GM-CSF (MP1–22E9). Foxp3 expression was assessed using the Foxp3/Transcription Factor Staining Buffer Set (eBiosciences). Flow cytometry was performed on a BD LSRFortessa™ (BD Biosciences) and analyzed using FlowJo software (FlowJo, LLC).

Treg suppression assay:

T effector (Teff) cells ($CD4^+CD25^-CD45.1^+$) were isolated (>95% purity) from spleens and lymph nodes of naïve CD45.1 congenic BALB/c mice by magnetic bead separation using a CD4 positive selection kit (StemCell) and subsequent depletion of $CD25^+$ cells ($CD25^+$ positive selection kit; StemCell). Treg cells ($CD4^+CD25^+CD45.2^+$) were purified (>95%) from spleens and lymph nodes of naïve BALB/c, SKG or $Nod2^{-/-}$SKG mice using EasySep™ Mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit II (StemCell). Teff cells (1×10^5/well) were pre-treated with 5μM of the cell proliferation dye Cell Trace Violet (ThermoFisher) and cultured with indicated numbers of Tregs, along with 2×10^5 CD45.1+ autologous BALB/c APCs that had been irradiated (1000 rads). Cultures were stimulated with 0.5μg/ml plate bound anti-CD3 (145–2C11, eBiosciences). Proliferation of Teff cells was assessed 72h post stimulation by cell trace violet dilution on the BD LSRFortessa™ (BD Biosciences) and analyzed using FlowJo software (FlowJo, LLC).

In vitro $CD4^+$ T cell stimulations:

$CD4^+$ T cells isolated from spleens and lymph nodes of 6 wk old, naïve SKG or $Nod2^{-/-}$SKG mice were purified (>95%) using EasySep™ Mouse CD4 negative selection kit (StemCell). Cells were seeded at 8×10^4 $CD4^+$ T cells/well into 96-well round bottom plates (Becton Dickinson) and resuspended in RPMI media containing 2 mM L-Glutamine

+10% FCS+50 ¼M 2-mercaptoethanol alone (unstimulated) or in media containing 20 ng/mL PMA and 1 ¼g/mL ionomycin. Supernatant was collected at 14h for measuring cytokine levels by ELISA.

Adoptive transfer:

Single cell suspensions were prepared from spleens and lymph nodes of 6 wk old, naïve donor SKG or Nod2$^{-/-}$SKG mice. CD4^{+} T cells were purified (>95%) using the EasySep™ Mouse CD4 positive selection kit (StemCell). Lymphopenic recipients (Nude or Rag1$^{-/-}$ mice) were i.v. injected with 2 × 10^{6} donor CD4^{+} T cells, and then were i.p. injected with 1.5 mg zymosan 24 h later.

Statistical analysis:

The non-parametric statistics test Mann-Whitney U test was used to compare 2 samples (e.g. zymosan-treated SKG to zymosan-treated Nod2$^{-/-}$SKG or BALB/c to Nod2$^{-/-}$BALB/c). Groups being compared are indicated within each panel. All data were analyzed using Prism (GraphPad Software, Inc.) and considered significant when $p<0.05$. Data are presented as box plots where the horizontal lines represent the median, boxes indicate the interquartile range, and error bars indicate the min and max or as bar graphs with the mean and standard error of the mean. All experiments were independently performed 2–3 times.

RESULTS

Nod2 is an important genetic determinant in T cell-mediated arthritis in SKG mice.

To investigate whether Nod2 plays a role in regulation of autoreactive T cells and pathogenesis of arthritis, we utilized an established T cell-mediated model of arthritis in SKG mice, and generated congenic SKG mice lacking *Nod2* (Nod2$^{-/-}$SKG). SKG mice sufficient and deficient in Nod2 were injected with β-glucans (zymosan) and monitored for development of arthritis (27, 28). By week 3 post zymosan injection Nod2$^{-/-}$SKG mice showed significantly worse clinical arthritis than SKG mice (Fig. 1A). Arthritis in Nod2-deficient animals worsened over time while disease in SKG mice remained relatively stable. Consistent with a prior report (27), SPF-housed SKG mice did not develop overt clinical arthritis in the absence of zymosan, a phenotype that was unchanged in Nod2-deficient SKG mice (Fig. 1A). Nod2$^{-/-}$SKG mice developed greater joint swelling, redness, and deformity within their forepaws, hindpaws and digits as compared to SKG mice (Fig. 1B; insets of clinical arthritis). Histopathological analysis of Nod2$^{-/-}$SKG mouse hindpaws and ankle joints revealed more extensive pathological changes, including substantial synovial hyperplasia within the sublining region that was marked by massive accumulation of mononuclear cells and pannus-eroded cartilage (Fig. 1B, boxes). Exacerbated “end-stage” destruction observed in ankles of Nod2$^{-/-}$SKG mice included fusion of subchondral bones and joint dislocations that are not typically present in SKG mice (Fig. 1B). In addition to their increased disease susceptibility, Nod2$^{-/-}$SKG mice experienced greater cumulative weight loss compared to their SKG counterparts (Fig. 1C).

To further evaluate how expression of Nod2 affects inflammation-associated molecular changes in cartilaginous and synovial joints, we applied near-infrared imaging (NIR)-

technology in combination with a fluorophore-labeled probe that is activated upon cleavage by proteases. Thus, the intensity of signal positively correlates with protease-mediated inflammation (28), a critical molecular response of arthritis. At 8 wk post- zymosan injection the joints from both Nod2$^{-/-}$SKG and SKG mice had greater overall signal intensity than naive BALB/c WT (Fig. 1D). Inflammatory protease activity was localized primarily to the ankle joint, but was also seen to extend from the knee to where the tibiotarsus is located to the ankle, as well as within the region of the tarso-metatarsal joints of the hindpaws (Fig. 1D, inset). Protease activity was significantly greater in the ankle, wrist, and knees of Nod2$^{-/-}$SKG mice than in SKG mice. Although inflammation was detected within the lower vertebral joints of the spine as reported (28), it was not significantly altered by *Nod2* deficiency (Fig. 1D). Taken together, these data indicate an endogenous protective function for Nod2 in mitigating joint inflammation caused by autoreactive T cells in SKG mice.

To determine whether Nod2 expression influences development of arthritis in the absence of the *skg* mutation, we injected WT (BALB/c) and Nod2$^{-/-}$ (BALB/c) mice with zymosan. By 8 wk, neither genotype had developed signs of arthritis as evaluated by clinical scoring or joint pathology (Fig. 2A). The more sensitive NIR-imaging detected a minimal but significant increase in inflammation within the ankle joints of WT mice compared to Nod2$^{-/-}$ mice (Fig. 2B). Development of sub-clinical synovitis has been reported as being T cell-independent (27), thereby underscoring the findings presented here suggesting the protective role of Nod2 in arthritis (Fig. 1) occurs through a T cell-mediated mechanism conferred by the *skg* mutation, rather than solely as a result of a response to zymosan.

In light of the sexually dimorphism of arthritis in SKG mice (28, 31), we next sought to rule out the possibility that the Nod2-associated arthritis phenotype in SKG mice was related to sex. Arthritis induced in male SKG and Nod2$^{-/-}$SKG mice (Supplementary Fig. 1) was found to be consistently worse in Nod2$^{-/-}$SKG mice compared to SKG counterparts, indicating that the role of Nod2 in controlling arthritis susceptibility is independent of sex.

Worsened arthritis in Nod2$^{-/-}$SKG mice is not the result of dysbiosis.

In addition to the effect of sex, animal-housing conditions (i.e. conventional vs. germ-free) and the presence of particular species of microbiota can influence the development of arthritis in SKG mice (24, 32). This information coupled with numerous studies suggesting that Nod2 is a critical regulator of the gut microbial composition and intestinal inflammation (33) we sought to evaluate whether worsened arthritis in Nod2-deficient mice would be affected by normalizing their microbiota to that of SKG mice. To do this SKG and Nod2$^{-/-}$ SKG mice were cohoused starting at the time of weaning (4wks of age) and after 4 weeks of cohousing arthritis was induced (Fig. 2C-H). Under cohousing conditions SKG and Nod2$^{-/-}$SKG mice received equal exposure to a composite of fecal and bedding material. Nod2$^{-/-}$SKG mice developed substantially increased clinical arthritis regardless of housing status (Fig. 2C). Furthermore, those cohoused with SKG mice experienced greater cumulative weight loss (Fig. 2D), similar to our findings of non-cohoused conditions (Fig. 1C). The worsened arthritis in cohoused Nod2$^{-/-}$SKG mice was corroborated by NIR-imaging (Fig. 2E) and by histopathological evaluation (Fig. 2F), which revealed severe ankle

inflammation, extensive synovial hyperplasia, and pannus-eroded cartilage similar to that observed in non-cohoused mice. Notably, clinical arthritis severity in SKG mice was the same, regardless of co-housing history (Fig. 2C), thereby supporting a protective function for Nod2 in arthritis independent from microbiota composition. These data are consistent with prior reports of cohoused SKG and BALB/c mice, wherein arthritis was neither horizontally transmissible nor altered by microbiota transfer (29).

To further evaluate the potential influence of microbiota on disease state, we examined the extent to which Nod2 influences the SKG-mediated intestinal disease previously described as ileitis (34), which is mediated by dysbiosis in SKG mice. The severity of ileitis coinciding with arthritis in SKG and Nod2$^{-/-}$SKG mice was assessed (Fig. 2G-H). In response to zymosan, the small intestine of SKG mice developed considerable inflammation of the ileum consisting of multiple, discrete segments of tissue distortion caused by a mixed mononuclear and polymorphonuclear cell infiltrate within the mucosal epithelium that in many cases extended into the lamina propria (Fig. 2G). Notably, SKG-mediated ileitis was almost completely abrogated in the context of Nod2 deficiency, suggesting that in the ileum expression of Nod2 is deleterious and contributes to ileitis in SKG mice. Collectively, data in Fig. 2 supports a protective function of Nod2 in mitigation of arthritis that is not likely a direct influence of dysbiosis or strain-specific microbiota.

Deletion of Nod2 augments the Th17 response in SKG mice.

To gain insight into the role of Nod2 in arthritic T cell responses, we evaluated the immune cell composition of lymph nodes that drain the ankle (popliteal) in arthritic SKG and Nod2$^{-/-}$SKG mice. Popliteal lymph nodes from arthritic Nod2$^{-/-}$SKG mice tended to have increased mass (Fig. 3A) and cellularity (data not shown) compared to those of SKG mice, though the differences were not significant in either case. However, we observed a significant increase in the total numbers of CD4^{+} T cells in the lymph nodes of arthritic Nod2$^{-/-}$SKG mice (Fig. 3B), suggesting expansion of a pathogenic T cell population. The expansion of CD4^{+} T cell responses was attributed to zymosan and the development of arthritis, as we have not observed differences in baseline T cell populations of untreated (naïve) mice (Supplementary Fig. 2). The increase in T cell numbers was also observed in the joint, as synovial fluid obtained directly from inflamed joints of arthritic Nod2$^{-/-}$SKG mice had increased numbers of T cells, including CD4^{+}, CD8^{+} and CD4^{-}CD8^{-}, in comparison to arthritic SKG mice (Fig. 3C). These data indicate that Nod2-deficiency results in increased numbers of T cells in response to zymosan that could promote arthritis.

Since CD4^{+} T cells that produce IL-17 (Th17 cells) are necessary for arthritis in SKG mice (26), we next examined whether Nod2-deficiency altered Th17-immunity. Intracellular cytokine staining of T cells from the popliteal lymph nodes showed an increase in the percentage and total numbers of IL-17-producing CD4^{+} T cells in arthritic Nod2$^{-/-}$SKG compared to arthritic SKG mice (Fig. 3D-F). Of note, the propensity of Nod2-deficient cells to produce IL-17 was observed in arthritic CD4^{+} T cells even without stimulation *in vitro* (Fig. 3E). Further evaluation of how Nod2-deficiency affected polarization of Th populations revealed that arthritic Nod2$^{-/-}$SKG also had increased proportions of Th1 cells along with greater proportion of CD4^{+} T cells producing additional cytokines including

GM-CSF and TNF, but not IL-22 (Supplementary Fig. 3). Further analysis specifically of the gated Th17 population demonstrated that the majority (~60%) of the Th17 cells co-produced TNF, along with smaller proportions co-producing GM-CSF, IL-22, and IFNγ (Fig. 3G), a phenotype that was genotype-independent. Consistent with the exacerbated Th17-response in Nod2$^{-/-}$SKG mice, levels of IL-17 (within ankle synovial fluid) were also significantly elevated in Nod2$^{-/-}$SKG vs. SKG mice (Fig. 3H). These data indicate that Nod2 deficiency results in increased generation of Th17 cells that potentiate arthritis development.

IL-17A is a critical factor in augmenting arthritis in Nod2$^{-/-}$SKG mice.

Given the above data indicating the importance of Nod2 expression in control of the Th17 response, we evaluated the contribution of IL-17 to the pathogenesis of arthritis in Nod2$^{-/-}$SKG mice. Arthritis was induced in Nod2$^{-/-}$SKG and SKG mice in concert with administration of anti-IL-17A blocking antibody or isotype control (Fig. 4). Consistent with a prior report (26), we observed an important role for IL-17 in induction of arthritis in SKG mice, as evaluated by clinical scoring (Fig. 4A), NIR imaging (Fig. 4B), and histopathology (Fig. 4C) of ankle joints, which demonstrated reduced overall inflammation in the anti-IL-17 treated, compared to untreated, SKG mice.. Importantly, Nod2$^{-/-}$SKG mice deplete of IL-17 exhibited significantly reduced arthritis, to the level of arthritic SKG mice (Fig. 4A). This finding was confirmed by NIR-imaging and histopathology that corroborated the reduction in joint inflammation in Nod2$^{-/-}$SKG mice (Fig. 4B,C). Collectively, these data indicate that increased IL-17 production is sufficient to cause worsened arthritis in Nod2-deficient SKG mice.

Nod2-deficiency does not alter CD4^{+} T regulatory function in SKG mice.

T regulatory (Treg) cells play an important role in suppression of autoimmunity, including negative regulation of inflammatory Th17 cells (35). Thus we sought to determine whether Nod2-deficiency altered Treg (CD4^{+}Foxp3^{+}) responses in SKG mice. The frequency of Tregs was indistinguishable between SKG and Nod2$^{-/-}$SKG mice regardless of whether they were in a naïve or arthritic state (Fig. 5A). Nod2$^{-/-}$SKG mice tended to have a slight, but not significant, increase in Treg frequency during arthritis compared to their naïve state. This could be a compensatory response to excessive inflammatory CD4^{+} T population in arthritic Nod2$^{-/-}$SKG mice. Nonetheless, there was no significant difference between genotypes, indicating that Nod2 expression does not overtly alter peripheral development of Tregs in SKG mice. We further evaluated whether Nod2-deficiency altered the suppressive function of Tregs in SKG mice. The ability of WT (BALB/c) effector T cells (CD4^{+}CD25^{-}) to proliferate *in vitro* upon activation by anti-CD3 and co-stimulation was evaluated in the presence of Tregs (CD4^{+}CD25^{+}) derived from naïve WT (BALB/c), SKG or Nod2$^{-/-}$SKG mice (Fig 5B). Consistent with a prior report (25) we found that SKG Tregs had impaired suppressive function (Fig. 5B). However, Nod2-deficiency did not further alter the suppressive function of SKG Tregs (Fig. 5B). These data indicate that the cellular mechanism by which Nod2 mitigates the Th17 response and arthritis is independent of Tregs.

Nod2 controls the ability of SKG CD4$^+$T cells to produce IL-17 and cause arthritis.

To gain further insight into the cellular mechanism by which the absence of Nod2 leads to more severe arthritis in SKG mice, we examined whether Nod2 influences inflammatory Th17 cell responses under homeostatic conditions (prior to treatment with zymosan). Indeed, we found that splenocytes from naïve (untreated) Nod2$^{-/-}$SKG mice had an increased frequency of Th17 cells compared to SKG mice (Fig. 6A). We next tested whether Nod2-deficient CD4$^+$ T cells produced more IL-17 on a per-cell basis. To do this we stimulated equal numbers of CD4$^+$ T cells purified from naïve SKG or naïve Nod2$^{-/-}$SKG mice with PMA/io. Indeed, CD4$^+$ T cells from naïve Nod2$^{-/-}$SKG mice produced greater amounts of IL-17 than SKG counterparts (Fig. 6B), indicating that Nod2-deficient SKG mice have increased Th17 responses under homeostatic conditions. In addition, when we examined the ability of purified CD4$^+$ T cells to produce other cytokines associated with Th1 and Th2 responses and found that in the absence of Nod2 production IFNγ was not altered and IL-10 production was reduced (Fig. 6C-D). Furthermore, zymosan did not have any direct effect on the ability of CD4$^+$ T cells to produce IL-17, IFNγ, or IL-10, as purified CD4$^+$ T cells stimulated with zymosan (50 μg/ml) did not result in detectable amounts of cytokine (data not shown). Cumulatively these data indicate a role for Nod2 as an endogenous suppressant of inflammatory Th17 responses.

We were next interested in determining if Nod2 affected the ability of pathogenic autoreactive T cells to cause arthritis on a single-cell level. To do this we adoptively transferred equal numbers of purified CD4$^+$ T cells from naïve Nod2$^{-/-}$SKG or SKG mice into lymphopenic nude recipients that were injected with zymosan 24h later and analyzed weekly for arthritis severity (Fig. 6E-G). Mice that received Nod2$^{-/-}$SKG CD4$^+$ T cells developed significantly worsened clinical arthritis than recipients of SKG CD4$^+$ T cells (Fig. 6E). The exacerbated clinical arthritis was corroborated by a marked increase in NIR-signal (Fig. 6F) and worsened histopathology that phenocopied data shown in arthritic Nod2$^{-/-}$SKG mice (Fig1B), including increased immune cell infiltrates and synovial hyperplasia (Fig. 6G). These data show that Nod2 is an intrinsic negative regulator of pathogenic autoreactive T cells.

To more thoroughly assess the T cell-intrinsic function of Nod2 in protection against arthritis in SKG mice, we adoptively transferred purified CD4$^+$ T cells from naive SKG (Nod2-sufficient) mice into Rag1$^{-/-}$ or Nod2$^{-/-}$Rag1$^{-/-}$ recipients, who were then treated with zymosan and evaluated for arthritis (Fig. 6H). Severity of arthritis was similar regardless of the recipient's genotype, indicating that lack of Nod2 expression by all cell types except T cells does not affect the pathogenicity of SKG CD4$^+$ T cells. These data further support a protective function for Nod2 within CD4$^+$ T cells as a suppressor of arthritogenic T cell responses and arthritis.

DISCUSSION

Nod2 is a critical regulator of immune homeostasis as mutations in *NOD2* cause the autoinflammatory disease Blau Syndrome, and confer significant risk for development of other inflammatory diseases including Crohn's disease, graft-versus-host disease and sarcoidosis (36). Despite the definitive importance of Nod2 in joint homeostasis as evident

in patients with Blau Syndrome, the cellular and molecular mechanisms by which Nod2 controls arthritis are unclear. Our studies provide insight into a novel role for Nod2 in regulation of T cell homeostasis as it relates to autoimmune forms of arthritis. We used the genetically susceptible strain of SKG mice, which upon exposure to β-glucans such as zymosan develop joint inflammation and pathology that is mediated by autoreactive $CD4^+$ T cells. Nod2-deficient SKG mice manifested an exacerbated form of arthritis that was accompanied by an increased magnitude of IL-17-producing autoreactive $CD4^+$ T cells, which were responsible for promotion of joint pathology. Interestingly, the affects of Nod2-deficiency on Th17 responses and arthritis were not due to altered Treg development or function, and occurred independently of sex or intestinal dysbiosis. Rather, Nod2-deficient $CD4^+$ T cells produced more IL-17 and had increased ability to cause arthritis on a single-cell level. Mechanistically, Nod2 did not appear to increase production of autoreactive T cells, as Nod2-deficient BALB/c mice (with the wildtype allele of ZAP-70) did not develop arthritis. Instead, naïve $Nod2^{-/-}$SKG mice had increased Th17 responses, indicating that Nod2 is an endogenous negative regulator of Th17 responses in SKG mice. These data identify a pivotal role of Nod2 in natural protection against autoimmune arthritis, in large part by homeostatic regulation of Th17 cell responses in SKG mice.

A key observation from this work is that the protective capacity of Nod2 occurred through control of autoreactive $CD4^+$ T cells. $CD4^+$ T cells purified from naïve $Nod2^{-/-}$SKG mice produced more IL-17 and had enhanced capacity to cause arthritis on a single cell level, indicating that endogenous Nod2 expression in T cells could directly suppress their arthritogenic capacity. A T cell-intrinsic role for Nod2 was previously established by Shaw *et al*. wherein Nod2-deficiency led to increased susceptibility of mice to *Toxoplasma gondii* infection due to dysregulated c-Rel activation within the T cell that resulted in a delay in IFNγ production that is essential to parasite clearance (10). Although IFNγ is known to suppress Th17 responses and arthritis in SKG mice (26), we and others did not see a change in the ability of $CD4^+$ T cells to produce IFNγ in Nod2-deficient mice (37). This discrepancy in IFNγ production could in part be explained by differences in the ways T cells were activated *in vitro* (antigen vs. CD3 vs. PMAio) or, in the case of our study, skewed by the presence of the *skg* mutation. Nonetheless, we interpret this data to mean that diminished IFNγ and/or Th1 response is not responsible for the enhanced Th17 responses of $Nod2^{-/-}$SKG mice.

The complexity surrounding Nod2 control over Th17 immunity is exemplified by the fact that Nod2 can either promote (38, 39), or repress (40, 41) the Th17 response, depending on specific factors within different scenarios (e.g. stimulus used). As with arthritis in SKG mice, there is evidence of negative regulation of Th17 immunity by Nod2 as a protective mechanism against intestinal disease; however, the cellular mechanisms appear to extend beyond T cell-mediated Nod2 function that includes intestinal epithelial cells (42). Our data are consistent with a prior report of T cell-induced enteropathy model, in which i.p. injection of anti-CD3 mAb was used to directly activate T cells *in vivo*, and the ensuing intestinal inflammation and Th17 response was worsened by Nod2-deficiency (42). Interestingly, using a different dose of anti-CD3 mAb with the same model system, another group reported the opposite observation in that Nod2-deficiency diminished both *il-17a* mRNA expression and intestinal disease (43). Collectively, we interpret this to mean that the role of Nod2 in

Th17 immunity is likely influenced by the kinetics of T cell activation and/or strength of TCR signaling, which is relevant in the context of arthritis in SKG mice that arises from impaired TCR signaling and generation of autoreactive T cells.

Autoreactive T cells that escape central tolerance in the thymus are subject to negative regulation by regulatory $CD4^+$ T cells, Tregs. We found that the expansion in Th17 cells was not likely due to diminished function of Tregs, as Nod2 deletion did not alter the peripheral development or suppressive function of Tregs in SKG mice. These data are supported by a previous study showing that Treg function is unchanged in Nod2-deficient C57BL/6 mice (44). Furthermore, we found that Nod2-deficient SKG $CD4^+$ T cells had impaired production of IL-10, a cytokine that directly dampens deleterious Th17 responses during inflammation and autoimmunity (45, 46). Nod2 has previously been demonstrated as a positive regulator of IL-10 production by myeloid cells (47, 48) and CD8 T cells (43) as part of a protective mechanism in intestinal disease. However, to our knowledge the ability of Nod2 to positively regulate IL-10 production by $CD4^+$ T cells and in the context of arthritis has not been described until now. Interestingly, autocrine production of IL-10 by self-reactive T cells has been proposed as a mechanism of self-regulation meant to suppress development of pathogenic autoreactive Th17 responses (49). These studies and our data support a potential function for Nod2 within $CD4^+$ T cells in dampening development of pathogenic Th17 cells. Since T cells express Nod2 during peripheral activation (9) as well as during thymic development (50), future studies that elucidate how Nod2 modulates arthritogenic T cell responses, which could arise from compromised peripheral and even central tolerance mechanisms will be of importance to our understanding of Nod2 in arthritis and autoimmunity.

Another interesting observation from this work is that different immunological mechanisms appear to underlie ileitis and arthritis in SKG mice, as exemplified by the discordant effects of Nod2 deficiency in the gastrointestinal tract ileum vs. joint. Whereas $Nod2^{-/-}$SKG mice developed worsened arthritis they had dramatically reduced ileitis compared to SKG mice, a phenotype that could be related to different roles for T cells and/or microbiota. The underlying mechanisms of ileitis development in SKG mice are continuing to be defined, but appear to be T cell-*independent* (34) and related to their inherent intestinal dysbiosis (32). In contrast, arthritis development in SKG mice is T cell-mediated (24) and independent of dysbiosis, as evidenced by cohousing studies of SKG mice with WT-BALB/c mice, which ameliorated induction of ileitis but not arthritis (29). Moreover, induction of arthritis was unaffected in germ-free mice (29), whereas ileitis was ameliorated. We would interpret our observations similarly to that of a recent report of compounding genetic interactions between Nod2 and development of colitis in predisposed SAMP1Yit/Fc mice (51). Given that distinct mechanisms pertaining to microbiota control of ileitis vs. arthritis occur in SKG mice would be further supported by our data demonstrating that worsened arthritis in $Nod2^{-/-}$SKG mice was maintained in mice cohoused with SKG mice. This data coupled with diminished ileitis in $Nod2^{-/-}$SKG mice would support an endogenous protective function of Nod2 in shaping T cell responses in arthritis, which is likely independent of microbiota composition.

Collectively, these findings illustrate how Nod2 may serve multiple mutually exclusive functions in pathways and tissues such as the gut vs. joints that can have varying, even

contrasting, effects on disease. This paradigm may offer new insight into interpretation of prior work by our lab and others that demonstrated promotion of arthritis by Nod2. In these models where arthritis is triggered by localized injury to the joint by PGN (20–23) or immune-complexes (52), a deleterious function of Nod2 was attributed to its MDP or PGN sensing function (20–23). In these scenarios the adverse mechanism of Nod2 is thought to be via the canonical Rip2 pathway that promotes synovial inflammation as mediated by innate cells such as neutrophils that are capable of producing IL-17 vs. alterations on effector T cell populations, or through mechanisms pertaining to promotion of adjuvant effects during immunization (53). In the synovial joint, Nod2 is expressed by several cells known to promote synovitis including fibroblasts and chondrocytes (13, 54), where its activation could potentiate local injury in a T cell-*independent* manner, as would be expected with mild arthritis we observed zymosan-injected WT-BALB/c mice (Figure 2A) and as reported (55). The protective effect of Nod2 in SKG mice required the *skg* mutation and could be transferred with Nod2-deficient SKG T cells, thereby underscoring the T cell-intrinsic mechanism of arthritis modeled here. Importantly, Nod2-sufficient SKG $CD4^+$ T cells were able to trigger arthritis in lymphopenic hosts (i.e. $Rag1^{-/-}$ or $Nod2^{-/-}Rag1^{-/-}$) irrespective of the recipient's genotype. These data indicate that the mechanism of protection of Nod2 in SKG mice originates from $CD4^+$ T cells rather than other cellular sources of Nod2 in the joint or periphery. Our studies reveal a novel role for Nod2 in T cell homeostasis that is independent of its role in microbial sensing or in acute inflammatory responses.

In conclusion, we present evidence for a unique function of Nod2 in attenuation of autoreactive T cell responses that cause arthritis. These data broaden our understanding of the biological functions of Nod2 to include roles in Th17 differentiation and T cell homeostasis that could help clarify the pathophysiology of arthritis. Understanding how Nod2 may participate in immune tolerance mechanisms could elucidate hitherto unappreciated mechanisms of protection that may be exploited for development of novel treatments for patients with Blau syndrome as well as other forms of arthritis such as RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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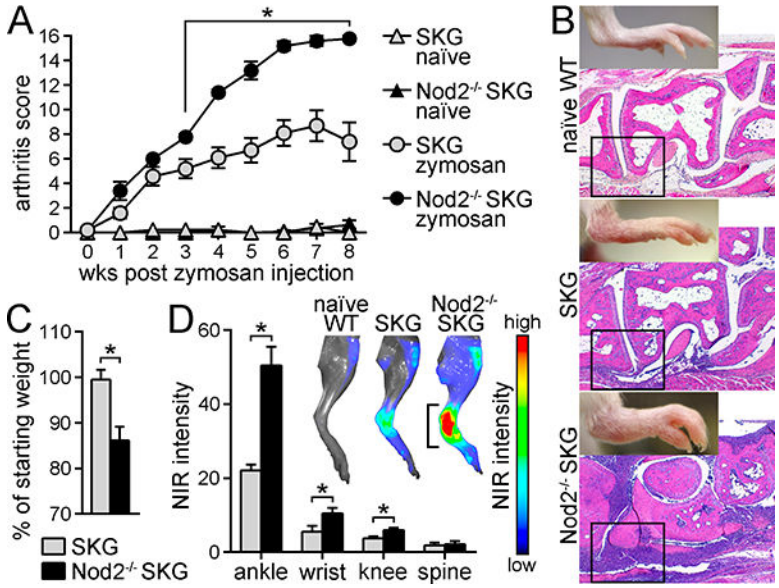

Figure 1. Nod2 deficiency worsens arthritis severity in SKG mice.

Arthritis was induced in SKG or Nod2$^{-/-}$SKG mice (6–7 wk of age) by a single i.p. injection of zymosan. (A) Clinical arthritis was scored weekly. (B) Photos of paws (insets) depicting arthritis in SKG (clinical grade 2) vs. Nod2$^{-/-}$SKG mice (clinical grade 4) compared to BALB/c WT (clinical grade 0). H&E-stained sections from ankle joints, with black boxes denote arthritic immunopathology. (C) Percent bodyweight loss from starting weight was determined for each mouse at 8 wk following zymosan. (D) Quantification (left panel) of the ankle, wrist, knee and spine and visualization (right panel) of signal intensity in the ankle (region indicated by bracket) in arthritic SKG or Nod2$^{-/-}$SKG mice at 8 wk post-zymosan using NIR imaging. The * indicates $p < 0.05$ between zymosan-injected SKG and Nod2$^{-/-}$SKG groups in all panels (Mann-Whitney U). Data are graphed as mean and standard error of the mean. Data are combined from 3 independently performed studies (n=24 mice/group).

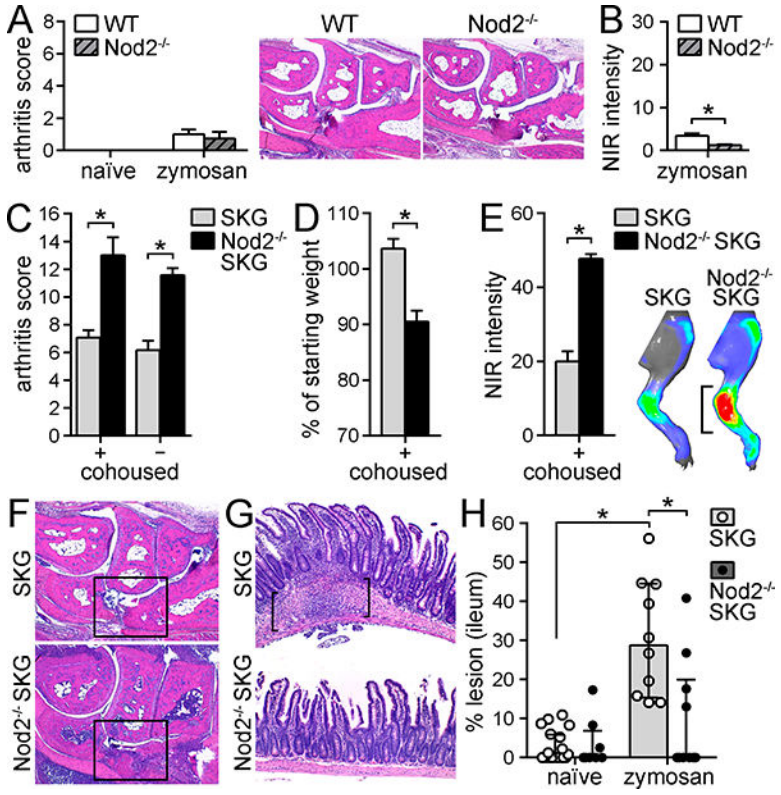

Figure 2. Nod2 mediated attenuation of arthritis in Nod2$^{-/-}$SKG mice is not the result of dysbiosis.

WT (BALB/c) and Nod2$^{-/-}$ (BALB/c) mice were injected with zymosan as described in Fig. 1. (A) Arthritis was evaluated weekly by clinical score and histopathology of the ankles at 8 wk post-zymosan injection. (B) Signal intensity within dissected ankles of WT vs. Nod2$^{-/-}$ mice was quantified by NIR-imaging at 8 wk following zymosan injection. (C-F) SKG or Nod2$^{-/-}$SKG mice were housed together "cohoused" (+) or housed separately "not co-housed" (–), beginning at the time of weaning (4 weeks) and throughout the duration of the experiment. Mice were evaluated at 8 wk following zymosan injection for (C) clinical arthritis severity and (D) percent bodyweight loss. (E) Quantification and visualization of signal intensity in ankles of arthritic SKG vs. Nod2$^{-/-}$SKG mice using NIR imaging. (F) H&E-stained sections of ankle joint, with black boxes denote arthritic immunopathology. (G-H) Small intestinal disease was evaluated in SKG vs. Nod2$^{-/-}$SKG mice at 8 wk following zymosan injection. (G) H&E-stained sections of the ileum depicting characteristic inflammation, e.g. massive mononuclear and polymorphonuclear cell infiltrate and tissue distortion underlying the mucosa, submucosa, and muscularis propria (bracket). (H) The % lesion per tissue was quantified within the ileum for each animal. Data are graphed as the median with interquartile range and combined from 2–3 independently performed studies. * indicates $p < 0.05$ (Mann-Whitney U). Data are representative of 3 independently performed studies (n=16–20 mice/group).

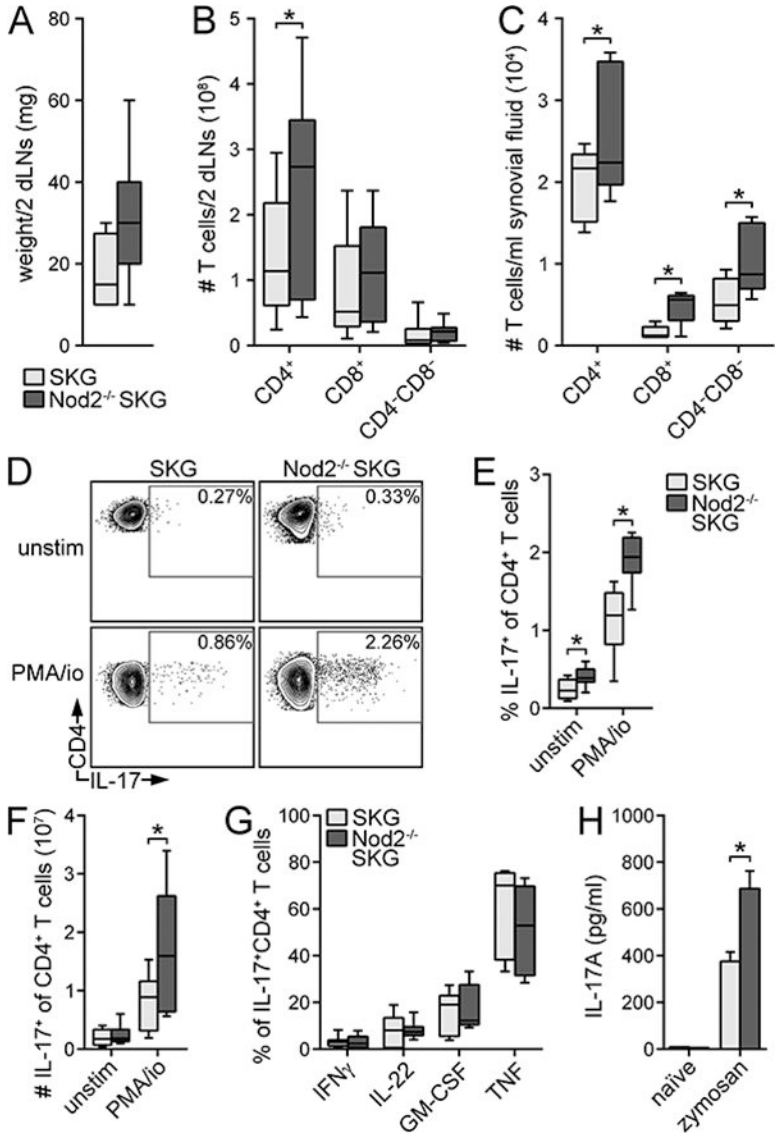

Figure 3. Nod2-deficiency results in increased T cellular responses and augmented Th17 immunity in arthritic SKG mice.

The T cellular response was evaluated 8 wk following injection of zymosan into SKG or Nod2$^{-/-}$SKG mice. (A) Combined weight of popliteal lymph nodes (2/mouse). (B) Quantification of the total number of live T cell subsets from the dLN by flow cytometry. (C) The number of live T cell subsets from synovial fluid aspirated from the sub-capsular space of ankle joints was quantified by flow cytometry. (D-G) Single cell suspensions from combined popliteal lymph nodes were stimulated *in vitro* with PMA/Ionomycin (PMA/Io), stained for intracellular cytokines, then analyzed by flow cytometry. (D) Dot plots showing frequency of IL-17-producing CD4^{+} T cells. Th17 cells were quantified and are shown as (E) percentage and (F) total number of live CD4^{+} T cells. (G) The Th17 cell population was gated and the frequency of Th17 cells co-expressing cytokines (IFNγ, TNF, GM-CSF, and IL-22) was quantified. (H) IL-17A protein in synovial fluid from the sub-capsular space of ankle joints 8 wk post-zymosan was quantified by ELISA. Data are graphed as median with

interquartile range. All data (expect panel H) are graphed as box and whisker plots demarcating the median with min to max whiskers and are combined from 2 independently performed studies. * $p < 0.05$ (Mann-Whitney U). Data in A-F are combined data from 2 independent experiments and data in (H) are representative of 3 independently performed experiments (n=10–12 mice/group).

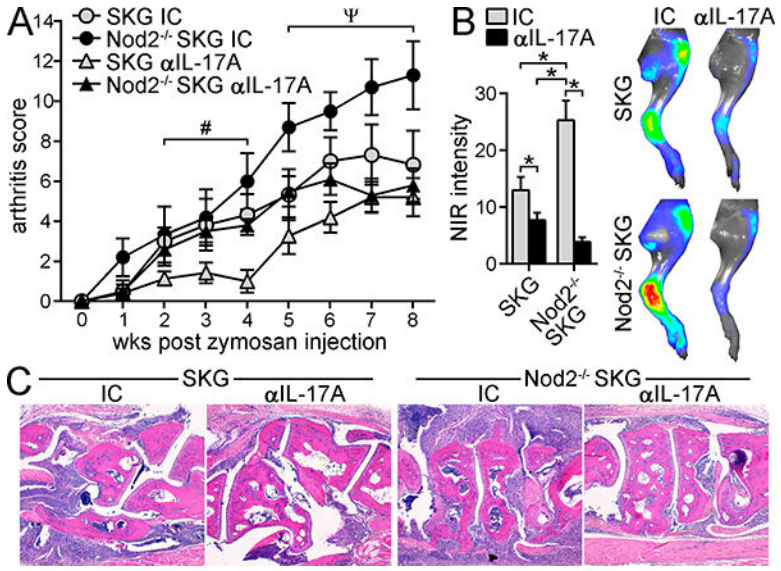

Figure 4. IL-17A is a critical regulator of arthritis in Nod2$^{-/-}$SKG mice.

SKG or Nod2$^{-/-}$SKG mice were administered α-IL-17A or isotype control (IC) antibody 24 h prior to receiving a zymosan injection and then weekly thereafter. (A) Severity of clinical arthritis and (B) quantification (left) and visualization (right) of signal intensity within ankles by NIR-imaging was determined at 8 wk post-zymosan. (C) H&E-stained sections of ankle joints (obtained 8 wk post-zymosan injection) showing the histopathological effects of IL-17A blockade for SKG vs. Nod2$^{-/-}$SKG mice. Data in panel A are combined from 2 independently performed studies (n=12 mice/group) and in panels B-C representative of 2 independently performed experiments. In panel A the # and Ψ indicate $p < 0.05$, comparison between αIL-17 and IC treatment of Nod2$^{-/-}$SKG mice. For other panels *$p < 0.05$ (Mann-Whitney U).

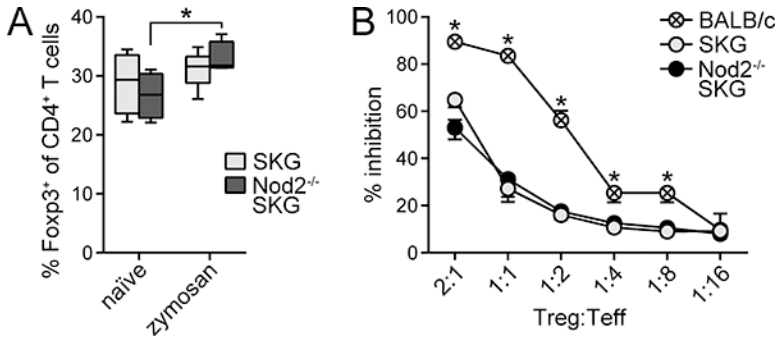

Figure 5. Nod2 is dispensable for peripheral Treg development and function in SKG mice.

(A) The frequency of Treg ($Foxp3^+$ cells) of total $CD4^+$ T cells in the popliteal lymph node was determined by ICS and flow cytometry in naïve or arthritic SKG and $Nod2^{-/-}$SKG mice at 8 weeks post zymosan injection. (B) The ability of indicated numbers of purified Treg ($CD4^+CD25^+$) cells from naïve BALB/c, SKG, or $Nod2^{-/-}$SKG mice to suppress proliferation of anti-CD3 stimulated Teff cells ($CD4^+CD25^-$) isolated from naïve BALB/c mice was determined by ICS and flow cytometry after a 72h co-incubation. *$p<0.05$ (Mann-Whitney) Data are representative of 2 independently performed experiments (n=3–6 mice/genotype/experiment).

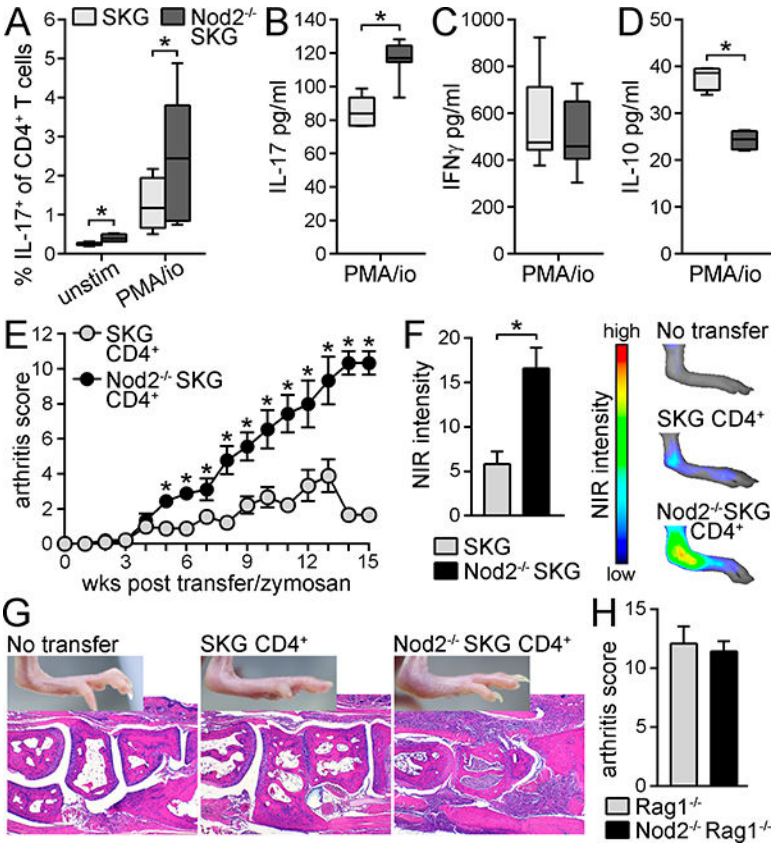

Figure 6: Nod2-deficient SKG CD4$^+$ T cells produce more IL-17 and have enhanced arthritogenicity on a single-cell level.

(A) The frequency of IL-17-producing CD4$^+$ T cells from spleen of naïve (i.e. non-zymosan-injected) mice in response to media or media + PMA/io was determined by ICS and flow cytometry. (B-G) Purified CD4$^+$ T cells from either naïve SKG or naïve Nod2$^{-/-}$SKG mice were stimulated (8×10^4/well) with PMA/io for 14hr and analyzed for secretion of (B) IL-17A, (C) IFNγ, or (D) IL-10 by ELISA or adoptively transferred (2×10^6) into nude recipients, who were 24h later injected with 1.5mg zymosan (E) scored weekly for severity of clinical arthritis, (F) assessed for inflammation by NIR imaging or (G) evaluated by histopathology. (H) Purified CD4$^+$ T cells from naïve SKG mice were adoptively transferred in equal numbers into either naïve Rag1$^{-/-}$ or naïve Nod2$^{-/-}$Rag1$^{-/-}$ mice and injected with zymosan 24h later. Severity of arthritis by clinical scoring was determined at 8 wk post zymosan. *p<0.05 (Mann-Whitney) Data are representative of 2–3 independently performed experiments (n=10–12 mice/group).