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Dectin-1 and NOD2 mediate cathepsin activation in zymosan-induced arthritis in mice

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Abstract

Objective—Activation of pattern recognition receptors (PRR) may contribute to arthritis. Here, we elucidated the role of NOD2, a genetic cause of inflammatory arthritis, and several other PRR in a murine model of inflammatory arthritis.

Methods—The roles of CR3, TLR2, MyD88, NOD1, NOD2, Dectin-1 and Dectin-2 were tested in vivo in arthritis elicited by intra-articular injections of zymosan, the fungal cell wall components curdlan, laminarin and mannan, and the bacterial cell wall peptidoglycan.

Results—Dectin-1, and to a lesser extent Dectin-2, contributed to arthritis. TLR2, MyD88 and CR3 played non-essential roles. Observations based on injection of curdlan, laminarin or mannan supported the dominant role of the Dectin-1 pathway in the joint. We demonstrated differential roles for NOD1 and NOD2 and identified NOD2 as a novel and essential mediator of zymosan-induced arthritis.

Conclusions—Together, Dectin-1 and NOD2 are critical, sentinel receptors in the arthritogenic effects of zymosan. Our data identify a novel role for NOD2 during inflammatory responses within joints.

Keywords

Arthritis models; NOD2; In vivo inflammation; Innate immunity; Zymosan

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Introduction

The etiology of most types of inflammatory arthritis remains unknown. It has long been observed clinically that systemic infections with many different pathogens can trigger a transient inflammatory arthritis, perhaps by activating receptors within the joint of the innate immune system. Indeed, experimental arthritis develops upon intra-articular injection of pathogen-associated molecular patterns (PAMPs), thereby demonstrating a role for innate immunity in joint inflammation. The innate immune system has evolved receptors called pathogen recognition receptors (PRRs), which are capable of recognizing and interacting with pathogens via their conserved PAMPs [1]. Given that PRRs are expressed within the rheumatoid synovium [2], they are likely mediators of initial responsiveness to PAMPs within the joint itself. Furthermore, a mutation in one specific PRR, nucleotide binding and oligomerization domain 2 (NOD2), is the cause of Blau syndrome, an autosomal dominant disease that causes inflammation in numerous organs including joints [3]. We have been investigating the role of NOD2 in normal joint biology and in animal models of inflammatory arthritis [4, 5]. Here, we report a novel role for NOD2 in zymosan-induced arthritis (ZIA).

The PRR system is an important non-self-recognition system which has evolved to recognize specific PAMPs. Toll-like receptor (TLR) family members are the most described PRRs, which act as crucial initiators of innate immune responses. While many studies have focused on TLRs in triggering joint inflammation, very few studies have addressed roles for “non-TLRs” such as the intra-cellular NOD-like receptors (NLRs) or C-type lectin-like receptors (CLRs). Since NLRs and CLRs are also pivotal sensors of infection [1, 6], they are just as likely to play roles in triggering joint inflammation. Indeed, many NLR members, including NOD1 and NOD2, are strongly linked with chronic inflammatory disorders that coincide with joint inflammation [6]. Furthermore, TLR and NLR pathways can intersect, providing enhanced responses through synergy or negative regulatory signals such as provided by NOD2 for several TLR responses [7, 8]. To explore a possible regulatory role for NOD2 within synovial joints, we studied zymosan-induced arthritis (ZIA) [9]. Prior studies have established that intra-articular injection of zymosan causes severe joint inflammation in mice that involves mononuclear cell infiltration, synovial hypertrophy and pannus formation with an early peak of disease at approximately 3 days and a later phase starting at 25 days [10]. Downstream inflammatory mediators such as inter-leukin (IL)-1, IL-6, tumor necrosis factor (TNF) and NO [11–14] along with aggrecanases and metalloproteinases (MMPs) [15] and transcriptional mediators such as STAT-1 [16] have been investigated in ZIA. However, very little work has been performed to understand the various innate immune receptors responsible for the initiation of ZIA.

Zymosan is a mixture of particles of varying size derived from cell wall fragments of *Saccharomyces cerevisiae* (*S. cerevisiae*). It is composed primarily of mannan, chitin and β-glucans. Zymosan-triggered inflammatory responses are known to involve production of cytokines, chemokines, lipid metabolites and reactive oxygen species (ROS) as well as indirect activation of complement and Fc-receptors by complement proteins and antibody opsonization, respectively [17]. Dectin-1 is a non-classical CLR and is considered the primary PRR for β-glucans such as curdlan [18]. As such, Dectin-1 plays a critical role recognizing zymosan and fungal pathogens. Dectin-1 is expressed predominantly by cells of myeloid origin, including macrophages, dendritic cells, monocytes and neutrophils, but is also expressed on a subset of T cells and epithelial cells [19–24]. Dectin-1 possesses an intracellular immunoreceptor tyrosinase activation motif (ITAM) in its cytoplasmic tail and during activation Src tyrosine kinase (Src) and spleen tyrosinase kinase (Syk) are activated [25]. Signaling downstream of Syk triggers phagocytosis, production of ROS, NF-AT and NF-κB [17]. Caspase recruitment domain-containing protein 9 (CARD9), complexed with

Bcl10 and MALT1, is a key intermediate activating NF-κB [26, 27]. Dectin 2 is a second CLR important for host defense against zymosan and fungi such as *Candida albicans (C. albicans)* [28, 29]; albeit it appears to recognize somewhat distinct ligands from that of Dectin-1.

In addition to engaging Dectin-1, fungal components are known to trigger other PRR, of which the TLR family has been studied most extensively. Although Dectin-1 is capable of signaling on its own in response to zymosan, engagement of TLR2 is also required for induction of inflammatory responses in macrophages studied in vitro. The extent to which receptors cooperate with Dectin-1 can vary amongst different cell types [18, 30]. Whether or not NLR family members, NOD1 and NOD2, cooperate with Dectin-1 or participate in responsiveness to fungal cell wall components has not been investigated. NOD1 and NOD2 belong to the NLR family and participate in recognizing peptidoglycans of bacteria [31]. Intriguingly, the signaling protein CARD9 is also known to interact directly with NOD2 and mediate some NOD2 responses [27], thereby suggesting potential cross-talk between the NOD2 and Dectin-1 pathways. Here, we sought to further elucidate the immune receptors through which zymosan is able to trigger arthritis in mice with emphasis on the role of NOD2.

Materials and methods

Reagents

Zymosan A and mannan isolated from *S. cerevisiae* along with laminarin isolated from *Laminaria digitata* were all purchased from Sigma-Aldrich. Mannan purified from *C. albicans* strain NGY335 was kindly provided by Dr. James Cutler (Louisiana State University Health Sciences Center, Louisiana). The remaining reagents were purchased commercially as indicated: curdlan (Wako Chemicals), rat anti-mouse Dectin-1 (2A11) antibody [32] and isotype control antibodies (ABD Serotec), rat anti-mouse Dectin-2 antibody (Accurate Chemical), peptidoglycan (PGN) from *Staphylococcus aureus* (InvivoGen), polymyxin B sulphate (Fluka). All reagents tested below the lower limit of detection of endotoxin activity.

Mice

Age- and gender-matched mice (8–12 weeks) deficient for TLR2, CR3, NOD1 and NOD2 and their congenic controls were purchased from Jackson Laboratories. Mice deficient in MyD88 were kindly provided by Dr. Shizuo Akira (Osaka University, Japan). Mice were housed in a facility approved by the Association of Assessment and Accreditation of Laboratory Animal Care International. All procedures were carried out in accordance with the National Institutes of Health and guidelines designated by Oregon Health & Science University Institutional Animal Care and Use policies.

Induction of arthritis

Under inhalation anesthesia with isofluorane, mice were administered an intra-articular (i.a.) injection of the indicated compounds as previously described in a 15–20 μl volume [5]: 140 μg zymosan, 140 μg curdlan, 200 μg mannan or 75 μg PGN. Reagents were dissolved in pyro-gen-free, sterile saline. As a control, the contralateral knee was administered an i.a. injection of equal volume of saline. For the induction of arthritis by zymosan or curdlan, these compounds were heated and/or homogenized by sonic emulsification at the time of injection as previously described [12]. For Dectin-1 or Dectin-2 blocking experiments, 15 μg of each antibody was co-administered with zymosan and/or curdlan within a 15 μl volume.

Near-infrared (NIR) fluorescence imaging of joint inflammation in vivo

The early events of inflammation within the joints involving protease activity were quantified as previously described [5] using Prosense (ViseEn Medical), a near-infrared (NIR)-fluorescent protease substrate that is incorporated into bone tissue and allows us to specifically visualize ongoing in vivo protease activity of cathepsins B, C, D, G, K, L and S, plasmin and plasma kallikrein. Data are represented as mean fold change in intensity relative to the saline controls.

Histology

Mouse knee joints were prepared for histological assessment as previously described [5]. Slides were photographed at 200× unless otherwise specified using a microscope (DM500B; Leica, Wetzlar, Germany) and a digital camera (CD500; Leica).

Statistical analysis

Data are represented as mean ± SEM. Mean differences between treatment and genotype controls were analyzed using a two-way and one-way analysis of variance with Bonferroni test or *t* test post-hoc analyses. Differences were considered statistically significant when $p < 0.05$.

Results

TLR signaling and CR3 are not essential for zymosan-induced arthritis

TLR2/6, TLR4 and the complement receptor, CR3, are believed to cooperate with Dectin-1 to promote certain cellular responses to zymosan. Using various knock-out (KO) mice, we tested the contribution of such receptors in ZIA. Consistent with prior reports, we find that inflammatory changes in the joint are apparent histologically at day 3 post i.a. injection of zymosan, in that increased mononuclear cell infiltration within the exudates is observed which is accompanied by massive synovial proliferation (Fig. 1). The indicated histological changes in response to zymosan coincide with increased signal within the joint as assessed by NIR-fluorescence imaging. NIR-fluorescence imaging with Prosense quantifies the amount of on-going cathepsin activation within the joint (Fig. 1a, b), which is considered an early event in the inflammatory cascade within the joint and has been used as a marker of arthritis [5]. At this point in time, we found that deficiency in TLR2 did not have a marked impact on ZIA severity based on NIR-imaging (Fig. 1b, left panel) or histology (Fig. 1c). Consistent with the intensity of signal captured by NIR imaging, zymosan-treated TLR2 KO mice showed a significant increase in the influx of inflammatory cells within the synovium and evidence of hypertrophy of synovial lining cells (Fig. 1c, center panel). We further assessed the contribution of TLR signaling events in mice lacking expression of the TLR adaptor molecule, MyD88. MyD88 KO mice developed inflammation to a similar extent as wild-type (WT) mice, as assessed by both NIR-fluorescence (Fig. 1b, middle graph) and histology (Fig. 1c). These data support a non-essential role for TLR signaling events in the induction of ZIA at day 3. ZIA was also investigated in CR3 KO mice and neither NIR imaging or histological assessments supported a statistically significant role for CR3 in zymosan-triggered arthritis. These data suggest that, while these receptors may be capable of promoting the release of mediators of inflammation in response to zymosan when isolated cells are studied, they are not essential for the induction of ZIA within joint tissue.

Dectin-1 is one of the main receptors responsible for ZIA

Dectin-1 has been identified as an essential mediator of the inflammatory effects of zymosan in primary macrophages studied in vitro [33]. To determine whether Dectin-1 participates in local joint inflammation in vivo, mice were administered an anti-Dectin-1 blocking antibody

or isotype control (I.C.) in combination with zymosan. We find that blockade of Dectin-1 markedly reduced inflammation as assessed by NIR imaging (Fig. 2a). The reduction in NIR intensity coincided with protection against ZIA histologically as anti-Dectin-1 blockade resulted in marked reduction in cell infiltration within the exudates or synovium of the joint (Fig. 2c, middle panel). At this point in time, minimal cartilage damage was observed histologically in mice administered the anti-Dectin-1 blocking antibody. However, we did note a mild synovial proliferation, indicating that Dectin-1 blockade does not completely abolish joint inflammation triggered by zymosan. Indeed, a statistically significant NIR response was observed for i.a. zymosan with anti-Dectin-1 antibody compared to joints injected with saline and I.C. antibodies (Fig. 2a).

Because Dectin-2 is implicated in cellular responses to fungi, we considered its role in ZIA. Mice were injected with zymosan in the presence of I.C. or anti-Dectin-2 blocking antibodies. We found that inhibition of Dectin-2 significantly diminished the zymosan-triggered NIR-fluorescence signal (Fig. 2b), which coincided with a diminished cellular response within the joint (Fig. 2c, bottom panel). However, anti-Dectin-2 antibody had a minimal effect on mononuclear cell infiltration compared to anti-Dectin-1. Indeed, we noted by NIR imaging that blockade of Dectin-2 (~40% reduction) did not diminish arthritis to the same extent as blockade of Dectin-1 (~65% reduction). These findings support a more dominant role for Dectin-1 over that of Dectin-2.

The β-glucan, curdlan, is a potent inducer of arthritis

Zymosan is a crude yeast cell wall extract consisting of particles of varying size containing chitin, β-glucans and mannan [34]. To assess which component(s) are capable of directly triggering arthritis, mice were administered i.a. injections of the β-glucans, curdlan (β-1,3-glucan) or laminarin (β-1,3-glucan with some branches of β-1,6 glucans), or mannan isolated from two different sources, *S. cerevisiae* and *C. albicans*. Administration of these compounds revealed that only curdlan was capable of triggering joint inflammation as judged by a significant increase in NIR fluorescence (Fig. 3a). Histological assessment of joint tissue following i.a. injections of laminarin or mannan did not reveal any pathological changes within the joint (data not shown), further indicating that they are non-arthritogenic. Indeed, we have administered up to 1,000 μg mannan in mice without any observable changes in joint inflammation as assessed by both NIR imaging and histology (data not shown), indicating that activation of the mannose receptor on its own is not a trigger of murine arthritis. We further showed that blockade of Dectin-1 completely abrogates curdlan-induced joint inflammation as assessed by NIR imaging (Fig. 3b). Consistent with the intensity of cathepsin activation by NIR imaging, histology of curdlan-treated mice showed a marked increased in infiltration of inflammatory cells within the synovium and the joint space (Fig. 3c, left panel), which was reduced in severity in the presence of anti-Dectin-1 blocking antibody (Fig. 3c, right panel). As a further demonstration of the specificity of curdlan for Dectin-1, we found that TLR2 KO mice do not show any altered joint inflammation (as assessed by NIR imaging) in response to curdlan (Fig. 3d, left panel).

Curdlan used in these studies is purified from the bacterium *Alcaligenes faecalis.* Therefore, to assess the possibility that contaminating lipopolysaccharide (LPS) could be responsible for the inflammatory effects of the curdlan preparation, mice were administered curdlan in the presence of polymyxin B (Fig. 3d, right panel). We found that polymyxin B has no effect on curdlan-triggered cathepsin activity indicating that inflammation induced by curdlan is not due to indirect activation of TLR4 by contaminating LPS but is a consequence of Dectin-1 activation. These data demonstrate that curdlan, which is considered the main constituent of zymosan, is responsible for the arthritogenic effect of zymosan via activation of Dectin-1.

A novel role for NOD2 as mediator of zymosan-arthritis

NLR family members NOD1 and NOD2 are known to be expressed in synovial membranes. While NOD1 and NOD2 play regulatory roles in TLR responses, their putative roles in modulating CLR responsiveness and/or sensing fungal cell wall components has not been investigated. Given the involvement of CARD9 in signaling pathways downstream of both the Dectin-1 and NOD2 receptors, there may be potential cross-talk between these two pathways. To determine the possible contribution of NOD2 in ZIA, NOD2 KO mice were administered i.a. injections of zymosan (Fig. 4). We found that deficiency of NOD2 strikingly reduced ZIA. NIR fluorescence triggered by zymosan was almost abolished in NOD2 KO mice (Fig. 4a). Consistent with NIR imaging, histopathology showed minimal joint inflammation. Very few inflammatory cells were observed within the synovium of NOD2 KO mice compared to WT controls (Fig. 4c, left and middle panels). At this point in time, the cartilage was well preserved in the NOD2 KO mice. We did, however, note that proliferation of the synovium (Fig. 4c, middle panel) was still increased over saline-injected controls, indicating that, like Dectin-1 blockade, the absence of NOD2 does not completely abolish all stimulatory pathways in ZIA. Another NLR family member, NOD1, is thought to signal via mechanisms homologous to NOD2 by way of RIP2. However, we did not find that NOD1 deficiency significantly altered the severity of ZIA based on NIR fluorescence (Fig. 4b). Increased mononuclear infiltrates and synovial proliferation were observed in NOD1 KO mice injected with zymosan (Fig. 4c, right panel). Thus, NOD2 is a newly identified mediator for zymosan-triggered arthritis.

In a previous report describing PGN-induced arthritis, we demonstrated that both TLR2 and NOD2 were involved in the development of arthritis [5]. A priori, there is nothing to suggest a role for Dectin-1 in PGN arthritis, so we used this model as a negative control for i.a. anti-Dectin-1 blocking antibody (Fig. 4d). As expected, we showed that PGN triggers arthritis independently of Dectin-1, indicting that anti-Dectin-1 antibody had no effect on an unrelated NOD2-dependent model of inflammatory arthritis.

Discussion

We used KO mice or inhibitors to identify the critical immune receptors responsible for zymosan-induced arthritis and cathepsin activity. Our results indicate that Dectin-1 is one of the major receptors responsible for ZIA. Dectin-2 played a lesser role and TLR2, MyD88 and CR3 played non-essential roles. Injection of purified fungal cell wall compounds including curdlan, laminarin and mannan support the dominant role of the Dectin-1 pathway. We also assessed the function of two NLR family members, NOD1 and NOD2, in ZIA. Our studies identify differential roles for NOD1 and NOD2 in ZIA. Surprisingly, NOD2 KO mice were protected against ZIA, thereby identifying NOD2 as a novel contributor to ZIA. These results thus establish functional, cooperative roles for NOD2 and Dectin-1 in a mouse model of inflammatory arthritis.

Our results identify an entirely novel role for NOD2 in amplifying inflammatory responses within the joint to zymosan. At this point, the mechanism by which NOD2 is involved in the joint's responsiveness to zymosan is unclear. NOD2 senses muramyl dipeptide as a structural component of bacterial PGN. Within the class of poly-saccharides classified as β-(1,3)-glucans, there are a number of structural variants. Glucans are the major structural components of the cell wall of fungi, but plants and certain bacteria such *Brucella*, *Agrobacterium* and *Rhizobium* are sources of β-glucans as well [35, 36]. We could speculate that NOD2 evolved to sense β-glucans on such bacteria or that molecular mimicry occurs between the structurally related β-glucans and peptidoglycan; however, this remains to be shown. Emerging reports have since expanded roles for NOD2 responsiveness to other agonists in the setting of viral [37] and toxoplasmotic [38] infections and to the

chemotherapeutic agent Vadimezan [39], thereby suggesting that NOD2 responsiveness extends to a broader range of agonists than previously appreciated. The apparent differences in structure amongst these agonists, however, would not support molecular mimicry or a direct interaction between NOD2 and its agonists, but rather a more downstream role as a signaling intermediary.

NOD2 has been shown to play a “fine-tuning” role in inflammatory models triggered by TLR activation, suggesting cross-talk between NOD2 and TLR pathways. For example, the response to cell wall fragments from *Streptococcus pneumoniae* induces both inflammatory and anti-inflammatory responses. The inflammatory responses are dependent on TLR2 and are NOD2-independent while the anti-inflammatory responses are dependent on TLR2 and NOD2, which involves IL-10 secretion and IL-10-regulated target gene expression [8]. The simultaneous activation of NOD2 by muramyl dipeptide (MDP) and TLRs by different ligands causes the TLR response to be downregulated in an interferon regulatory factor 4-dependent manner [7]. The cross-talk is thought to occur through converging pathways leading to NF-κB activation. Since our data here do not support essential roles for MyD88 or TLR2, we conclude that the contribution of NOD2 in this model is probably not due to any influence on TLR signaling. Indeed, our prior report supports the independence of NOD2 and TLR2 signaling in arthritis [5].

Whether or not NOD2 pathways can intersect and alter the function of zymosan-activated Dectin-1 pathways within the joint has not been previously addressed until this report. There are numerous sequellae of Dectin-1 activation, including the generation of ROS, release of chemokines, cytokines, anti-inflammatory cytokines and inflammatory lipid mediators, and induction of phagocytosis (reviewed in [17]). Testing the role of NOD2 in fine-tuning any one of these response may be informative. It is interesting to speculate that cross-talk between NOD2 and Dectin-1 may occur via CARD9, an essential downstream mediator of Dectin-1, which also directly interacts with NOD2 [27]. CARD9 was found to be important for NOD2-dependent activation of p38 and JNK pathways, but not NF-κB activation [27]. Studies are underway to test the hypothesis that NOD2 and Dectin-1 pathways converge at CARD9 in response to zymosan and upregulate various inflammatory mediators in the joint.

The mechanisms involved in responsiveness to zymosan in vivo are complex. Even for cell culture models showing zymosan responses that are dependent on TLR2, the component of zymosan that is detected by TLR2 has not been definitively identified, although several glycolipids are candidates [17]. Dectin-1 recognizes zymosan and β-1,3-linked glucans with the minimal unit being a 10-mer [40], as well as whole fungi such as *C. albicans* and *S. cerevisiae*. Among the various components of zymosan we tested in isolation for their ability to trigger arthritis, the β-(1,3) glucan, curdlan, demonstrated the most inflammatory potential within the joint. Curdlan-induced arthritis was abolished by co-treatment with anti-Dectin-1 blocking antibody. Laminarin has been shown to exhibit a high degree of specificity for binding to Dectin-1, but is not thought to activate Dectin-1 [41]. Local injection of mannan did not result in any measurable joint inflammation, indicating that activation of the mannose receptor on its own is not sufficient for induction of arthritis. These data, combined with the anti-Dectin-1 blocking experiments, support our conclusion that Dectin-1 is one of the primary receptors involved in ZIA. Notably, activation of Dectin-1 by curdlan alone did not result in the same extent of arthritis as zymosan did, again suggesting a role for other receptors capable of detecting zymosan.

Our data on TLR2-deficient mice are consistent with previous reports where no differences were noted in ZIA in TLR2-deficient mice 3 days after i.a. injection [42]. However, our report does describe differences in arthritis at day 1 and day 24, suggesting a role for TLR2 very early and very late in disease. The lack of a role for TLR2 at day 3 may seem surprising

given evidence that some zymosan-induced inflammatory responses in macrophages in vitro are abrogated in TLR2 or Myd88-deficient cells [17]. However, at the level of the intact animal and in specific organs, receptor density, tissue distribution and differential expression of other participating molecules such as NOD2 may play a more significant role. Cellular composition and architecture in each individual organ may lead to different inflammatory responses from those exhibited in homogeneous cells in isolation. For example, while we find zymosan responses in the joint to be independent of TLR2 but dependent on Dectin-1, administration of zymosan directly into lungs by intratracheal aerosolization triggers inflammation independent of both TLR2 and Dectin-1 [43], indicating that different organs handle localized exposure to zymosan in unique ways and other receptors participate in the recognition of zymosan. Systemic exposure of autoimmune-prone mice to zymosan has also revealed striking differences in disease pathology. Zymosan, via Dectin-1, potentiates arthritis in SKG mice, a strain with a genetic predisposition to produce arthritogenic autoreactive T cells [44]. Surprisingly, treatment of non-obese diabetic (NOD) mice, which are genetically prone to autoimmune type I diabetes, with zymosan results in suppression of autoimmunity [45]. The mode of zymosan delivery, the particular organ exposed, and the underlying specificities of host T cells are factors that influence the outcome of both T-cell-based models of autoimmunity and models of inflammation based on activating PRRs.

Other C-type lectin receptors such as Dectin-2 have been shown to recognize zymosan and carbohydrate moieties of fungi. Like Dectin-1, the signal transduction pathway of Dectin-2 also involves Src kinases, CARD9 and activation of NF-κB. However, unlike Dectin-1, Dectin-2 lacks an intracellular ITAM motif and does not initiate signals but couples with Fc receptor gamma (FcRγ) chain signaling. We found that blockade of Dectin-2 with a neutralizing antibody did significantly diminish ZIA, but not to the same extent as we observed with the blocking studies for Dectin-1. We are aware that a caveat to these studies is the potencies of the blocking antibodies, which could be a variable as opposed to examination of Dectin-1 and Dectin-2 function using KO mice where complete inhibition would be achieved. NOD1 does not appear to play any significant contribution to ZIA. Despite the two NLRs activating homologous signal transduction pathways downstream of RIP2 and NF-κB, such differential roles for NOD2 and NOD1 in response to other agonists have been reported [46, 47]. This would suggest that there are unique or opposing functions for NOD1 versus NOD2 responses.

Although the mechanism by which NOD2 and Dectin-1 modulate zymosan-triggered arthritis is not entirely clear at this point, their relevance is supported by our in vivo studies which demonstrate that NOD2 and Dectin-1 are both required for ZIA. It will be of interest to determine whether NOD2 plays a role in zymosan responses in other organs or if this is a joint-specific response. Unraveling the role of NOD2 in inflammatory models may provide insights into NOD2's function in normal joint homeostasis and provide insights into the mechanism of arthritis seen in patients with NOD2 mutations.

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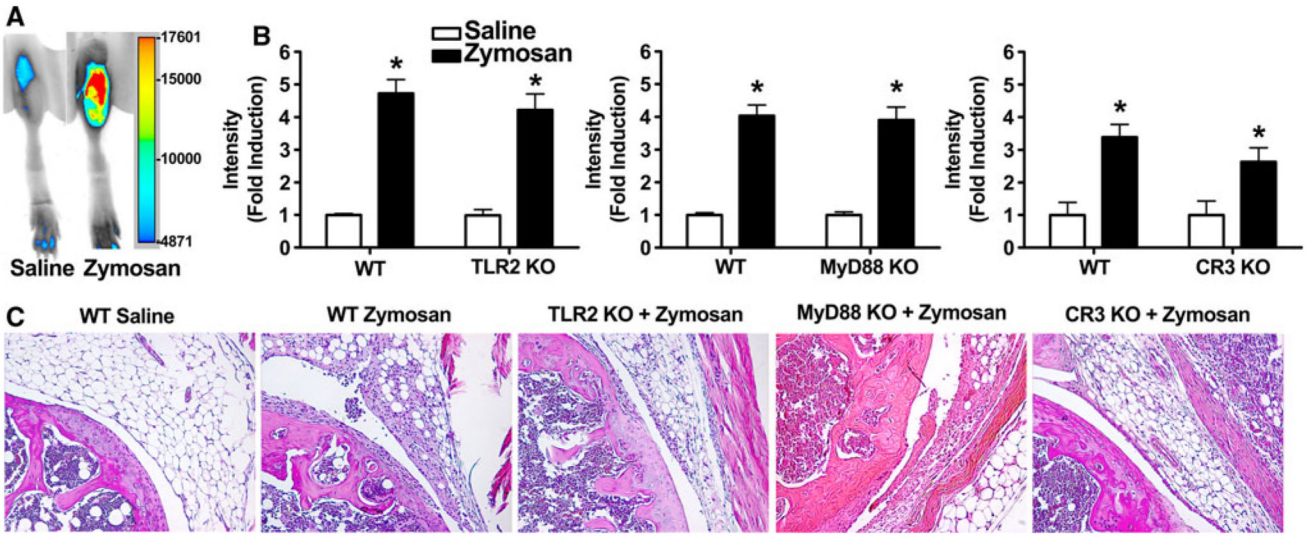

Fig. 1.

TLR signaling and CR3 are not essential for zymosan-induced arthritis. Mice were administered an i.a. injection of 140 μg zymosan or saline in the contralateral knee and inflammation within the knee joint was assessed 3 days later. **a** Representative NIR-fluorescence images of knee joints from WT mice treated with saline vs zymosan; **b** Quantification of mean NIR intensity (i.e. cathepsin activity) in TLR2 KO mice (*left*), MyD88 KOs (*center*) and CR3 KOs (*right*) versus WT controls, **c** Histological assessment of knee joints of the indicated KO mice. Data are the mean ± SEM; $*p < 0.05$, comparison between zymosan and saline treatments within a genotype; $^{\Psi}p < 0.05$ comparison between KO and WT mice treated with zymosan (n = 6–10 mice/treatment/genotype)

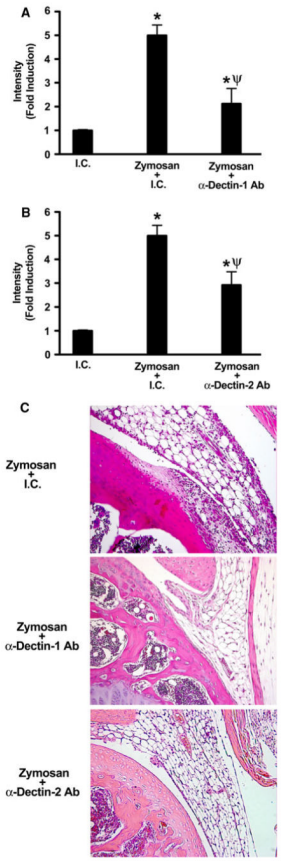

Fig. 2.

Dectin-1 is one of the main receptors responsible for zymosan-induced arthritis. Mice were administered an i.a. injection of 140 μg zymosan in the presence of anti-Dectin-1 blocking antibody or isotype control (I.C.) and inflammation within the knee joint was assessed 3 days later. **a** Quantification of mean NIR intensity (i.e. cathepsin activity) of the effect of anti-Dectin-1 antibody on ZIA; **b** Quantification of mean NIR intensity of the effect of anti-Dectin-2 antibody on ZIA; c Histological assessment of knee joints following injections of zymosan in the presence of either I.C. (*top panel*), anti-Dectin-1 (*middle panel*) or anti-Dectin-2 (*bottom panel*) antibodies. Data are the mean ± SEM; $*p < 0.05$, comparison between zymo-san + I.C. and saline + I.C. treatments; $^{\Psi}p < 0.05$ comparison between anti-Dectin-1 or anti-Dectin-2 antibodies and I.C. treatments in the presence of zymosan ($n = 6–9$ mice/treatment)

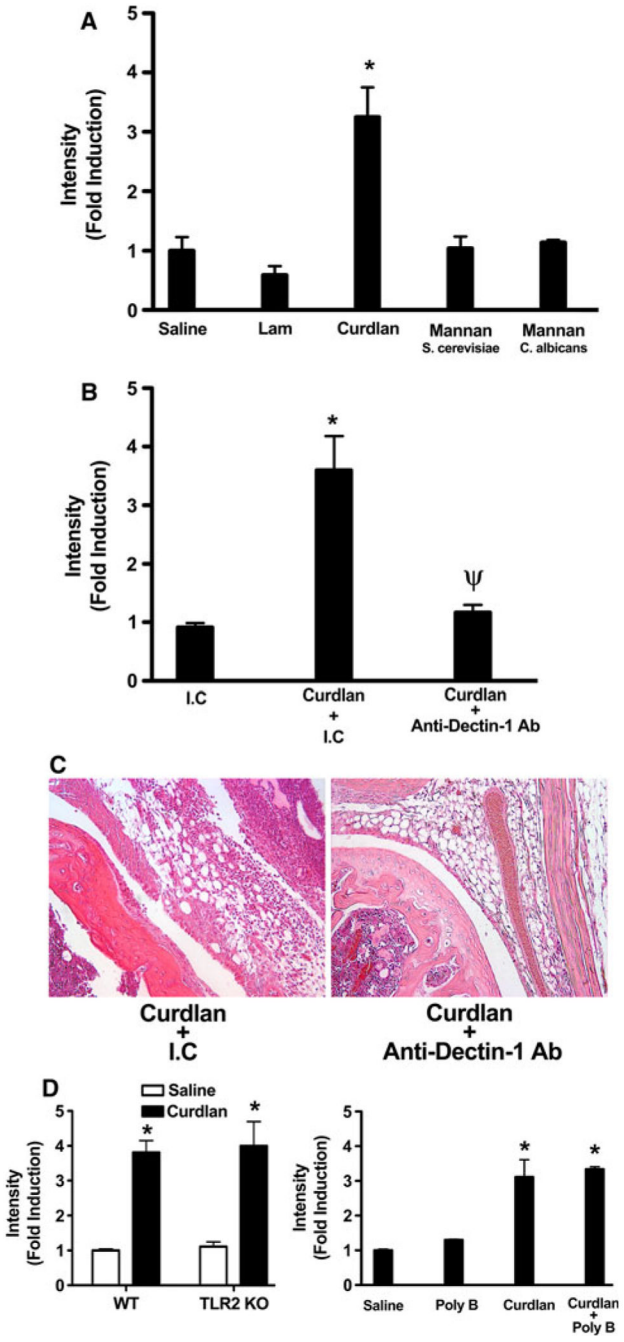

Fig. 3.

The β-glucan, curdlan, is a potent inducer of arthritis. Mice were administered an i.a. injection of 140 μg curdlan or 200 μg of laminarin or mannan isolated from either *S. cerevisiae* or *C. albicans*. Inflammation within the knee joint was assessed 3 days later by NIR imaging (**a**). Curdlan was administered in the presence of anti-Dectin-1 blocking antibody or I.C. and knee joint inflammation was assessed 3 days later by NIR imaging (**b**) or by histology (**c**). **d** Quantification of NIR imaging (i.e. cathepsin activity) 3 days post i.a. injection of curdlan in TLR2 KO mice versus WT controls (*left panel*); or in response to co-injection of curdlan and polymyxin B sulphate (*right panel*). Data are the mean ± SEM; *$p <$

0.05, comparison versus saline-control treatment; $^{\Psi}p < 0.05$ comparison between anti-Dectin-1 antibody and I.C. antibody treatments in the presence of zymosan ($n = 6–8$ mice/treatment/genotype)

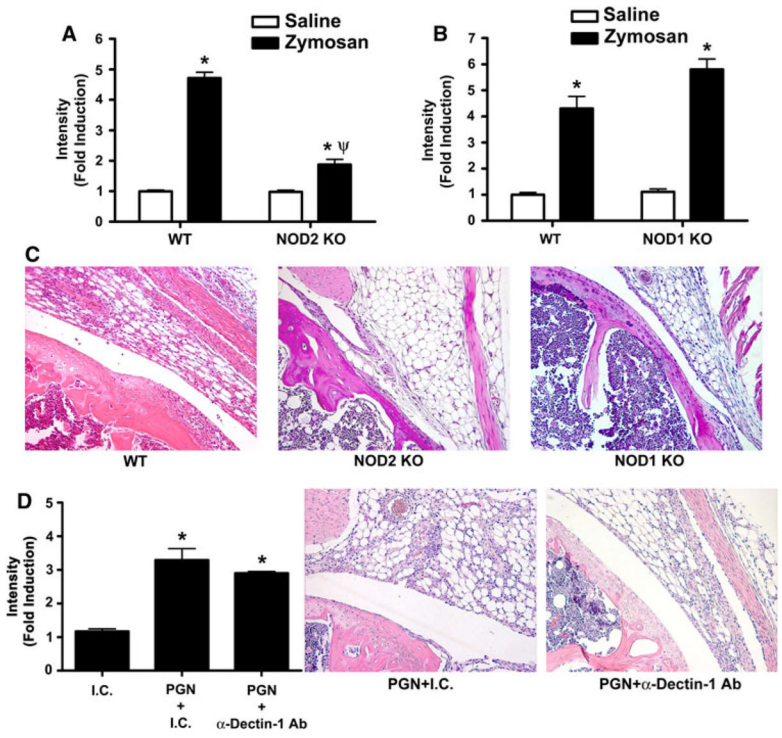

Fig. 4.

A novel role for NOD2 as an essential mediator in zymosan-arthritis. Mice were administered an i.a. injection of 140 μg zymosan or saline in the contralateral knee. Inflammation within the knee joint was assessed 3 days later. Quantification of mean NIR intensity (i.e. cathepsin activity) in NOD2 KO mice (**a**) or NOD1 KO mice (**b**) versus WT controls. **c** Histological assessment of knee joints of the indicated KOs. **d** Quantification of mean NIR intensity of the effect of anti-Dectin-1 blocking antibody on PGN-induced arthritis (*left panel*) and histological assessment of knee joints following injections of PGN in the presence of either I.C. or anti-Dectin-1 antibodies (*middle and right panels*). Data are the mean ± SEM; $*p < 0.05$, comparison between zymosan and saline treatments within a genotype; $^{\Psi}p < 0.05$ comparison between KO and WT mice treated with zymosan (n = 6–9 mice/treatment/ genotype)