NOD1 deficiency impairs CD44a/Lck as well as PI3K/Akt pathway

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Pattern recognition receptors (PRRs) are crucial for host defense and tissue homeostasis against infecting pathogens. PRRs are highly conserved cross species, suggesting their key roles in fundamental biological processes. Though much have been learned for NOD1 receptor in the innate and adaptive immune responses, the roles of NOD1 during embryonic and larval stages remain poorly understood. Here, we report that NOD1 is necessary for the modulation of PI3K-Akt pathway and larval survival in zebrafish. Transcriptome analysis revealed that the significantly enriched pathways in NOD1⁻/⁻ zebrafish larvae were mainly involved in metabolism and immune system processes. Biochemical analysis demonstrated that NOD1 was required for the expression of CD44a that, in turn, activated the PI3K-Akt pathway during larval development. Conversely, over-expression of CD44a in NOD1-deficient zebrafish restored the modulation of the PI3K-Akt pathway and improved larval survival. Collectively, our work indicates that NOD1 plays a previously undetected protective role in larval survival through CD44a-mediated activation of the PI3K-Akt signaling.

NODL-like receptors (NLRs) are a large family of intracellular pattern recognition receptors (PRRs) with a characteristic arrangement of nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) regions. The most prominent function of NLRs is to recognize intracellular pathogen-associated molecular patterns (PAMPs)³. Phylogenetic analysis has revealed 3 distinct subfamilies within the NLR family: the NODs (NOD1-2, NOD3/ NLR3, NOD4/NLR5, NOD5/NLRX1, CIITA), the NLRPs (NLRP1-14, also called NALPs) and the IPAF subfamily consisting of IPAF (NLRC4) and NAIP6,7. Of them, NOD1 is the first characterized NLR member⁴. In mammals, NOD1 senses D-glutamyl-meso-diaminopimelic acid (DAP), a fragment of bacterial peptidoglycan (PGN) found in all Gram-negative bacteria and a few Gram-positive bacteria⁵,6. Upon ligand binding, NOD1 interacts with receptor-interacting protein 2 (RICK/RIP2) through CARD-CARD domain interaction, which triggers the activation of NF-κB and MAPK (mitogen-associated protein kinase) pathways⁷,⁸. These signaling cascades potently up-regulate the production of pro-inflammatory cytokines and antimicrobial molecules, constituting the innate immune response.

Significant advances have been achieved regarding the function of NOD1 signaling pathway, including its role in innate immune responses to bacterial and protozoan parasite infections⁹,¹⁰, in shaping adaptive immune responses towards bacteria and bacterial-derived constituents¹¹,¹², and in autophagy and inflammatory signaling in response to invasive bacteria¹³,¹⁴. NOD1 signaling was also reported to be involved in insulin resistance in mammalian adipocytes¹⁵–¹⁷. In contrast, very little is known about the role and mechanisms of NOD1 in early stage of ontogeny, although one recent report found that the activation of NOD1 signaling induced fetal growth restriction and death in mice¹⁸.

NOD1 genes have been cloned from rainbow trout¹⁹, goldfish²⁰, orange-spotted grouper²¹, olive flounder²², channel catfish²³,²⁴, rohu²⁵ and grass carp²⁶. The teleost NOD1 has a structure with signature conserved domains similar to the mammalian counterpart. Experiments entailing loss-of-function or gain-of-function studies indicate that piscine NOD1 is crucial for the expression of pro-inflammatory cytokines¹⁶,²² and a shared antibacterial function²³,²⁷. In zebrafish, morpholino-mediated depletion of NOD1 does not affect embryonic development and larval survival rate, even in response to bacterial infection. However, the splice-blocking morpholino may not penetrate target cells at late stages of zebrafish larvae²⁷. Whether piscine NOD1 contributes to immune defense and relevant mechanism during early embryonic development and larval survival remains to be resolved.

CD44 is a transmembrane adhesion molecule and the major receptor for hyaluronan. In addition to the crucial role of mediating T-cell extravasation²⁸,²⁹, regulating effector T-cell responses³⁰ and T-cell development³¹, CD44 also activates the PI3K-Akt pathway which is associated with cell survival in various cell types including

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leukemia and T-cell. NLRs and Toll-like receptors (TLRs) have shaped our current understanding of innate regulation of adaptive immunity. Recent studies have identified a cross talk among CD44, TLRs and the PI3K-Akt pathway in pathological conditions. However, whether the functional correlation among NOD1, CD44 and PI3K-Akt pathway exists in the immune system, especially during early ontogenesis, is still unclear.

Our previous report showed the high expression of NOD1 in the embryonic and larval stage of zebrafish. This promoted us to generate NOD1−/− zebrafish, to establish whether NOD1 deficiency affects hatching process and larval survival in the early ontogenesis, and to determine the possible molecular mechanisms. In addition, we conducted rescue experiments to investigate the correlation between NOD1 and CD44 receptors. The current study highlights NOD1 is essential for CD44a-mediated activation of the PI3K-Akt pathway and zebrafish larval survival.

**Results**

**Generation of NOD1 knockout zebrafish using the Cas9/gRNA system.** Previous studies have shown that the Cas9/gRNA system efficiently executes site-specific cleavage, and is a highly effective and scalable gene knockout method in zebrafish in vivo. To study the function of NOD1, we employed the Cas9/gRNA system to generate NOD1 knockout zebrafish. A gRNA with 23-bp “target sequence” was designed, which starts with two GG residues at the 5’ end for efficient transcription from the T7 promoter and ends with the protospacer adjacent motif (PAM) NGG at the 3’ end, which is indispensable for Cas9 binding and cleavage. Cas9 mRNA and NOD1 gRNA were microinjected into one-cell embryos of zebrafish. The results from sequencing of PCR fragments from a single zebrafish about 2 months old revealed two or more peaks at the same location. As expected, the Cas9/gRNA-mediated mutations occurred at or near the target site. A group of representative mutations was presented in Fig. 1b, including insertions of 1-2 base-pairs (NOD1-1IS−/− and NOD1-2IS−/−). We further produced homozygotic NOD1-1IS−/− mutants through self-fertilization of heterozygotes that was confirmed by sequencing (Fig. 1c).

To confirm the deletion of NOD1 in zebrafish by western blotting, we generated an anti-NOD1 monoclonal antibody. Antibody specificity was verified by immunoblotting against transfected NOD1-FLAG or another NLR protein NOD2-FLAG. NOD1 antibody detected a strong band corresponding to the exogenous FLAG-tagged NOD1 (left in Fig. 1d). Using the NOD1-FLAG construct as a positive control, we detected a protein of similar size in wild-type (WT) zebrafish larvae. These results clearly demonstrate that NOD1 antibody specifically detect endogenous NOD1 protein in zebrafish (right in Fig. 1d). After confirming the specificity of zebrafish NOD1 antibody, we examined the effect of the NOD1-1IS−/− mutation on NOD1 protein expression by western blotting in NOD1 WT and knockout zebrafish larvae. The band corresponding to WT NOD1 protein (of ~100kDa) was not detected in homozygous NOD1-1IS−/− mutants (left in Fig. 1e). This indicates that the Cas9/gRNA-mediated mutation ablates the expression of NOD1 in zebrafish.

**Loss of NOD1 in zebrafish impairs embryo hatching and larval survival.** Our previous report concerning the constitutive expression of NOD throughout zebrafish development suggests that NOD genes have important functions in developmental processes. Hatching is a critical period for zebrafish embryogenesis. We thus evaluate the effect of NOD1 in zebrafish hatching process. At 2 days post-fertilization (dpf), only 4.9% of the WT zebrafish embryos hatched out of the chorion, and no statistical difference existed between the WT and NOD1-1IS−/− zebrafish. At 3 dpf, over 90% of the WT zebrafish embryos hatched out of the chorion, whereas NOD1-1IS−/− zebrafish embryos hatched at a significantly decreased rate of 28.9% (Fig. 2a and b). At 4 dpf, all the WT and NOD1-1IS−/− zebrafish embryos hatched out of the chorion, and the hatching rate increased to 84.4% in the NOD1-1IS−/− zebrafish embryos, indicating a delayed hatching process (Fig. 2b). Therefore, the pivotal period of NOD1 affecting embryo hatching happened at 3 and 4 dpf. Notably, NOD1 deficiency also impacts the embryo survival rate, and the deaths occurred mainly within the first day after fertilization (Fig. 2c).

The hatched larvae from WT and NOD1-1IS−/− zebrafish were used for survival analysis. We found that survival of the NOD1-1IS−/− zebrafish, compared to WT zebrafish, was similar during 1~5 days post-hatching (dph), slightly decreased at 6 dph, and greatly reduced at 7~9 dph (Fig. 2d). The death of NOD1-1IS−/− zebrafish peaked roughly at 5~8 dph. Overall, the survival rate of NOD1-1IS−/− zebrafish is significantly lower than that of WT zebrafish (Fig. 2d). Taken together, our results show that NOD1 deficiency in zebrafish impairs embryo hatching and larval survival.

**Comparative transcriptome analysis reveals that NOD1 deficiency affects larval metabolism and immune system processes.** To investigate the possible mechanism that NOD1 impacts larval survival in zebrafish, we performed transcriptome analysis to explore NOD1-related signaling pathways. Zebrafish larvae from WT and NOD1-1IS−/− were collected at 10 dpf (7 dph), when the survival rate of WT and NOD1-1IS−/− zebrafish larvae started to diverge significantly. The results showed that a total of 863 out of 40137 genes were considered to be differentially expressed (363 upregulated and 500 downregulated) when p value ≤0.05 and [logFC] ≥1 were set as the cut-off limits (Fig. 3a and b). Based on NR annotations, the Gene Ontology (GO) classification system was used to classify the possible functions of differentially expressed genes. A total of 272 genes (31.52%) were successfully assigned to at least one GO term annotation. For the molecular function category, the top two largest categories were “binding” and “catalytic activity” (Fig. 3c). We also performed an enrichment analysis of the KEGG pathways for all differentially expressed genes. The significantly enriched pathways were mainly involved in metabolism including “Metabolism of other amino acids”, “Xenobiotics biodegradation and metabolism”, “Lipid metabolism”, “Metabolism of cofactors and vitamins”, “Energy metabolism”, and involved in immune system including “Antigen processing and presentation” and “NOD-like receptor signaling pathway” (Supplementary Table S1).
Comparative transcriptome analysis showed that 31 NLRs containing NACHT and LRR domains were differentially regulated by NOD1 (Fig. 4a). Since the expansion of NLR-encoding genes has been described in the zebrafish 41, 42, we further examined the phylogenetic relationship of these differentially expressed NLRs and NODs subfamily. The result of phylogenetic analysis showed these differentially expressed NLRs regulated by NOD1 were mainly the homologues of NOD3 (Fig. 4b). 22 NLR genes were also selected and used for qRT-PCR validation. PCR amplification showed that all qRT-PCR primers produced single fragments of the expected lengths (109~410 bp). Except for gene2604, gene1133, gene33648, gene26693, gene1131, gene1132 and
gene12961, the expression of other 15 NLRs was in agreement with their transcript abundance changes determined by RNA-seq (Fig. 4c and a). Some data is not very consistent between RNA-seq and qRT-PCR, such as gene1133 and gene26693, which may be due to different batch samples or different calculation method for measuring gene expression. Besides NLRs, NOD1 also regulated the expression of tripartite motif proteins (15 DEGs) and immunity-associated proteins (17 DEGs) (Supplementary Fig. S1).

Comparative transcriptome analysis showed that a total of 10 genes involved in “Antigen processing and presentation” pathway, which include calreticulin (gene18050), major histocompatibility complex class I-related gene protein-like (gene34465), major histocompatibility complex class I UBA gene (gene30491), HLA class I histocompatibility antigen, B-47 alpha chain (gene30489), class I histocompatibility antigen, F10 alpha chain-like isoform X1 (gene34464), novel protein similar to MHC class II beta chain (gene18954) and 4 Hsp70 genes (gene3968, gene3967, gene3966 and gene17831), were differentially regulated by NOD1 (Fig. 4d). Except for calreticulin, the expression levels of other 9 DEGs were significantly weakened in NOD1−/− zebrafish larvae.

Although adaptive immunity is absent in the early zebrafish embryo and not functionally mature until at least 2 to 3 weeks postfertilization43, zebrafish larvae within 10 dpf have a constitutive expression for the above mentioned 5 MHC related genes and mhc2dab (gene18822)44. To further confirm the transcriptional regulation of MHC class I and II genes, the expression patterns of 6 selected MHC related genes were further studied in zebrafish embryo and larvae by qRT-PCR. Unexpectedly, the expression of similar to mhc1uba (gene30489) was up-regulated at NOD1−/− zebrafish larvae, whereas the expression of mhc1uma (gene34464), mhc1ula (gene34465), mhc1uba (gene30491), MHC-II3 (gene18954) and mhc2dab (gene18822) were greatly attenuated at NOD1−/− zebrafish embryo and/ or larvae (Fig. 4e). These results show that NOD1 regulates the expression of MHC class I and II genes involved in “Antigen processing and presentation”.

Previous study showed that mammalian NOD1 was critically involved in the activation of NF-κB and MAPK4. However, comparative transcriptome analysis demonstrated that the conventional NF-κB and MAPK immune signaling pathways were normal in the NOD1−/− zebrafish larvae. To confirm the unchanged activities of NF-κB and MAPK, we performed qRT-PCR for NF-κB and MAPK target genes by using RNA from WT and NOD1−/− zebrafish larvae at 10 dpf. Most target genes including nfkib1, nfkbiaa, nfkbiab, MAPK1, MAPK7, MAPK8, MAPK10 and MAPK14 remained unchanged, except for the decreased expression of nfkib2 and MAPK3.
(Supplementary Fig. S2). These results show that NOD1 deficiency did not significantly affect NF-κB and MAPK pathways at the transcriptional level.

**PCR arrays and western blotting reveal that NOD1 is critical for CD44a/Lck as well as PI3K/Akt pathway.** The above results indicate that NOD1 deficiency affects larval immune system. Since 32% inconsistent expression exists between RNA-seq and qRT-PCR, we further validate the expression difference of innate and adaptive immune genes between WT and NOD1-1IS−/− zebrafish larvae collected at 10 dpf by our previous custom RT2 Profiler PCR arrays. This array contains a total of 372 genes involved in Type I interferon response, Toll-like receptor signaling, JAK/STAT signaling, T-Cell & B-cell activation, antibacterial and antiviral responses. The array analysis revealed that 11 genes were up-regulated and 15 genes were down-regulated in NOD1-1IS−/− zebrafish. Among them, NALPL1 and CD44a were the top two down-regulated in NOD1-1IS−/− zebrafish, with a decrease in expression of 122- and 1534-fold, respectively (Fig. 5a). The tyrosine kinase Lck, which associated with CD44, was also down-regulated in NOD1-1IS−/− zebrafish (Fig. 5a). Since the expression tendency of NALPL1 and CD44a from PCR arrays was inconsistent with transcriptome data, we further examined the expression of NALPL1 and CD44a at more time points with more biological replicates. The results of qRT-PCR showed that NOD1 deficiency did impair the expression of NALPL1 and CD44a both at zebrafish embryo and larvae (Fig. 5b).

Mammalian CD44 is a widely-expressed adhesion receptor, and involved in activating the PI3K/Akt signaling pathway, which is associated with cell survival in various cell types. Comparative transcriptome analysis showed...
Figure 4. NOD1 is critical for innate and adaptive immunity during larval development. (a) The gene cluster for "NOD-like receptors". At the top of this panel, a color key denotes the gradient scale of gene expression from low (green) to high (red) degrees. (b) Phylogenetic tree of NOD-like receptors. Phylogenetic relationships were based on amino acid alignments. Bootstrap values based on 10,000 replicates are indicated on each branch. The evolutionary history was inferred using the neighbor joining method. All positions containing gaps and missing data were eliminated from the dataset (pairwise deletion). Accession numbers of other NLR sequences from NCBI databases are as follows: zebrafish NOD2, NP_001314973; zebrafish NOD3, XP_003201049; zebrafish NLRC5, XP_003200494; human NLRC5, NP_115582; human NOD1, AAD28350; human NOD2, AAG33677. (c) Validation of transcriptome sequencing results by quantitative RT-PCR. Those genes with the same expression tendency between RNA-seq and qRT-PCR are in red. Data represent the mean ± the SEM, and were tested for statistical significance using two-tailed Student's t-test. **p < 0.01. (d) The gene cluster for "Antigen processing and presentation". At the top of this panel, a color key denotes the gradient scale of gene expression from low (green) to high (red) degrees. (e) The expression changes of MHC class I and II genes in the NOD1-1IS−/− zebrafish compared with WT. 30–50 embryos or larvae from WT and NOD1-1IS−/− zebrafish were collected at 2, 3, 5, 7 and 10 dpf, and used for qRT-PCR. Data represent the mean ± the SEM, and were tested for statistical significance using two-tailed Student's t-test. **p < 0.01.
Figure 5. NOD1 deficiency impairs CD44a-PI3K-Akt pathway. (a) Heatmap analysis for PCR-arrays data from WT and NOD1-1IS/− zebrafish at 10 dpf. The custom RT² Profiler PCR arrays containing 372 genes involved in Type I interferon response, Toll-like receptor signaling, JAK/STAT signaling, T-Cell & B-cell activation, antibacterial and antiviral responses were applied. Three biological replicates were performed for the WT and NOD1-1IS/− zebrafish. (b) The expression changes of CD44a and NALPL1 in the NOD1-1IS/− zebrafish compared with WT. 30~50 embryos or larvae from WT and NOD1-1IS/− zebrafish were collected at 2, 3, 5, 7 and 10 dpf, and used for qRT-PCR. Data represent the mean ± the SEM, and were tested for statistical significance using two-tailed Student’s t-test. **p < 0.01. (c) Heatmap analysis for PCR-arrays data from WT and NOD1-1IS/− zebrafish at 10 dpf. Zebrafish PI3K-Akt Signaling Pathway RT² Profiler PCR Array and mTOR Signaling RT² Profiler™ PCR Array were applied. Three biological replicates were performed for the WT and NOD1-1IS/− zebrafish. (d) Immunoblot analysis of phospho-Akt, Akt, phospho-S6, S6, phospho-GSK3β, GSK3β and phospho-CREB in larvae homogenate from WT and NOD1-1IS/− zebrafish at 10 dpf. Western blotting results were quantified using Quantity One software. Data represent the average of two independent experiments. *p < 0.05, **p < 0.01.
that 11 genes involved in PI3K/Akt signaling pathway were differentially regulated by NOD1 (Supplementary Fig. S3a). To determine whether NOD1 impacts larval survival through CD44a-mediated modulation of the PI3K/Akt signaling, we evaluated the effect of NOD1 on the activation of PI3K and Akt downstream of mTOR. We employed two PCR arrays to profile the PI3K-Akt and mTOR signaling cascades. Zebrafish larvae from WT and NOD1-1IS−/− collected at 10 dpf were used for Zebrafish PI3K-Akt Signaling Pathway RT² Profiler PCR Array and mTOR Signaling RT² Profiler™ PCR Array. Among 84 genes involved in the PI3K-Akt pathway, the expressions of pdk1, tlr4bb, itgb1b and PIK3R2 were significantly decreased, whereas that of eif2ak2 was significantly increased in NOD1-1IS−/− zebrafish compared to WT zebrafish. Within the 84 genes involved in the mTOR pathway, transcripts of akt2l, ddit4, rps6ka2 and rragd were reduced in NOD1-1IS−/− zebrafish, with that of akt2l was the most pronounced (79-fold) (Fig. 5c). To further corroborate with the gene induction of these two pathways, we probed the protein expressions of Akt and several key substrates thereof, including GSK3β, S6 and CREB, by western blotting. The result demonstrated that loss of NOD1 inhibited the expression of Akt, GSK3β and S6. The phosphorylation levels of Akt, GSK3β, S6 and CREB were also decreased in NOD1-1IS−/− zebrafish collected at 10 dpf (Fig. 5d). The decreased levels of Akt and phosphorylated GSK-3β were observed at earlier time points such as 5 and 7 dpf. The expression of p-Akt, GSK3β and S6 in NOD1-1IS−/− zebrafish was also decreased at 7 dpf compared with WT zebrafish (Supplementary Fig. S3b). These results indicate that loss of NOD1 results in the inhibition of PI3K-Akt signaling.

CD44a is critical for NOD1-mediated regulation of PI3K-Akt, but not for NOD1-mediated regulation of MHC class I and II genes. During embryonic and larval development, many MHC class I and class II molecules, such as mhc1uma, mhc1ula and MHC-IIβ, have similar impaired expression pattern with CD44a in NOD1-1IS−/− zebrafish (Fig. 4e and Fig. 5b). Further, the overexpression of CD44a in WT zebrafish could induce the expression of these MHC class I and class II molecules, such as mhc1uma, mhc1ula, similar to mhc1uba and mhc2dab (Fig. 6a). To determine whether CD44a is required for the defective expression of MHC related genes in NOD1-1IS−/− zebrafish, rescue experiments were performed by overexpression of CD44a in NOD1 knockout zebrafish. The impaired expression of endogenous CD44a in NOD1-1IS−/− zebrafish could be rescued by exogenous CD44a (Fig. 6b). However, the expression of exogenous CD44a failed to restore the expression of MHC related genes in NOD1-1IS−/− zebrafish (Fig. 6c). These results show that CD44a expression is not sufficient for NOD1-mediated MHC genes expression, suggesting that other unknown signaling pathways are required for MHC gene expression downstream of NOD1.

We next tested whether the decreased PI3K-Akt signaling in NOD1-1IS−/− zebrafish was due to the impaired expression of CD44a. Overexpression of CD44a was found to be effective until 7 dpf (13-fold, Supplementary Fig. S4a) and could restore CD44a expression in NOD1-1IS−/− zebrafish at 7 dpf (Fig. 6b), we thus examined the PI3K-Akt signaling cascade from WT and NOD1 knockout zebrafish with or without CD44a overexpression at 7 dpf by western blotting. In WT zebrafish, CD44a overexpression increased the levels of phosphorylated and total proteins of Akt and GSK3β (Fig. 6d). Furthermore, overexpression of CD44a in NOD1-1IS−/− zebrafish rescued the expression of phosphorylated and unphosphorylated Akt, but not for GSK3β, an enzyme that regulated glyco-gen synthesis (Fig. 6e). Taken together, these results suggest that NOD1 regulates Akt expression through CD44a.

CD44a overexpression in NOD1-1IS−/− zebrafish rescues larval survival. Having shown that CD44a overexpression rescued Akt expression in NOD1-1IS−/− zebrafish, we wanted to know whether CD44a overexpression could rescue larval survival. The hatched larvae from WT and NOD1-1IS−/− zebrafish microinjected with control or CD44a-FLAG construct were used for survival analysis. Compared with WT zebrafish microinjected with the control plasmid, no obvious difference was observed for WT microinjected with the CD44a-FLAG plasmid up to 14 dpf (p = 0.8407), and significant divergence of survival curves observed for NOD1-1IS−/− zebrafish microinjected with the control (p = 0.0004) or CD44a-FLAG construct (p = 0.0082) (Fig. 7a). Since we noted that CD44a was not sufficiently overexpressed in zebrafish larvae at 12 dpf (corresponding to 8 dpf), a statistically significant difference of the survival curves lasting for 8 dpf were again observed using the Log-Rank Test. As shown in Supplementary Fig. S4b, no significant divergence of survival curve was observed between WT zebrafish microinjected with the control plasmid and NOD1-1IS−/− zebrafish microinjected with the CD44a-FLAG construct (p = 0.0683). However, NOD1-1IS−/− zebrafish microinjected with CD44a-FLAG had a higher survival rate than NOD1-1IS−/− zebrafish microinjected with the control construct, with the observed significance level (p = 0.0056) (Supplementary Fig. S4b). This result shows that NOD1 impacts larvae survival via CD44a.

Discussion

Although the in vivo relevance of NOD1-mediated signaling for immunity against various pathogens including bacteria, virus and parasites has been clearly demonstrated43,45, the role of NOD1 during developmental processes has not been explored in detail. In the present study, we demonstrate that zebrafish NOD1 is required for hatching process and larval survival. The present study demonstrates that NOD1 is a multifunctional regulator that drives the expression of multiple receptors and immune signaling pathways. The present study also confirms the crucial role of NOD1 in larval survival through a CD44a-mediated PI3K-Akt signaling cascade.

Multiple studies have identified positive or negative regulatory functions of NLRs in innate immune responses. Studies of gene deletion or knockdown show that NLRP6 impedes the clearance of both Gram-positive and -negative bacterial pathogens through negatively regulating MAPK and canonical NF-κB pathways47, while NLRP12 is a negative regulator of inflammatory T cell responses and T cell-mediated disease48. NLRC3 negatively regulates innate immune signaling induced by the DNA sensor STING49. In line with aforementioned studies, NLRC5 and NLRX1 attenuate innate immune responses by inhibiting the NF-κB and type I interferon pathways50,51. NOD2 is necessary for the NF-kappaB/IL-1-beta-mediated innate responses against bacteria challenge.
Figure 6. NOD1 regulates Akt expression through CD44a. (a) The effect of CD44a overexpression in WT zebrafish on the expression of MHC class I and II genes. (b) The expression of CD44a in WT and NOD1-1IS−/− zebrafish microinjected with p3 × FLAG or CD44a-FLAG. (c) The expression of MHC class I and II genes in NOD1-1IS−/− zebrafish. For (a–c), all samples were collected at 7 dpf and used for RNA extraction and qRT-PCR (mean ± SEM, n = 3). The WT zebrafish microinjected with p3 × FLAG were used as control. Data were tested for statistical significance using two-tailed Student’s t-test. ***p < 0.001, **p < 0.01, *p < 0.05. (d) Immunoblot analysis of Akt, GSK-3β, phospho-Akt and phospho-GSK-3β in larvae homogenate from WT zebrafish microinjected with p3 × FLAG or CD44a-FLAG (n = 50–60 larvae each group) at 7 dpf. (e) Immunoblot analysis of Akt, GSK-3β, phospho-Akt and phospho-GSK-3β in larvae homogenate from WT and NOD1-1IS−/− zebrafish at 7 dpf microinjected with p3 × FLAG or CD44a-FLAG (n = 50–60 larvae each group). For (d–e), western blotting results were quantified using Quantity One software. Data represent the average of two independent experiments. *p < 0.05, **p < 0.01.
in Crohn’s disease. These findings highlight a broad spectrum of functions of NLRs in regulating innate immune responses. MHC class I and II pathways play essential roles in the activation of adaptive immune responses. Among NLR members, NLRC5 and CIITA are key transcriptional regulators of MHC class I and II genes, respectively. Interestingly, we showed that NOD1 is required for the expression of both MHC class I and II genes in vivo. The present study identified NOD1 as a new regulator to drive the expression of MHC class I and II genes. Further studies are needed to clarify the mechanism by which NOD1 transcriptionally regulates the expression of MHC class I and II genes, and if NOD1 plays redundant or exclusive roles with NLRC5 and CIITA in MHC class I- and class II-dependent immune responses.

NLRs, Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) are three families of pathogen sensors. Much progress has been made on how these innate sensors are activated to initiate host innate immune signaling. It has become increasingly apparent that there are extensive interplays among these families of pattern recognition receptors. Various levels of crosstalk between NLRs and TLRs, and between NLRs and RLRs have been described. NOD1 and NALPL1 belong to the members of NLR family. Our previous work showed that overexpression of NALPL1 contributed to protection against bacterial and viral infection. Moreover, NALPL1 overexpression induced the expression of NOD1. The impaired expression of NALPL1 in the NOD1-1IS−/− larvae reported here suggests that NALPL1 and NOD1 constitute a feed-forward circuit in host immune defense. Comparative transcriptome analysis also screened multiple differentially expressed NLRs regulated by NOD1. Our unpublished data showed that these NLRs regulated by NOD1 positively or negatively regulated innate immune responses.

Figure 7. CD44a overexpression in NOD1-1IS−/− zebrafish rescues larval survival. (a) Survival of WT and NOD1-1IS−/− zebrafish microinjected with p3×FLAG or CD44a-FLAG (n = 44–120 larvae each group). The survival curves lasting for 14 dph were compared statistically significant difference using the Log-Rank Test. (b) Proposed model illustrating the intracellular signaling pathways modulated by NOD1 during zebrafish larval development at homeostasis. p, phosphorylation.
immune signaling, which suggested that the interplays between NOD1 and other NLRs may contribute to the maintenance of immune homeostasis.

It is well accepted that CD44 is a major cell surface receptor for the extracellular matrix glycosaminoglycan hyaluronan, and serves as a coreceptor for other receptors such as epidermal growth factor receptor (EGFR)/ErbB1 and ErbB2 [Her2]34, c-Met35, TGF3 receptors 1 and 236. CD44 prevents exaggerated inflammatory responses to LPS by promoting the expression of negative regulators of TLR4 signaling37. It also directly associates with TLR2 which negatively regulates TLR-mediated inflammatory responses38 and participates in IP-10 induction39. In addition, ligation of CD44 can activate the PI3K-Akt signaling pathway that is critical for cell survival of various cell types33, 66, 67. Therefore, it is significant to find the modulation of zebrafish NOD1 on the CD44a and PI3K-Akt signaling. Furthermore, the expression of exogenous CD44a in zebrafish embryos had no effect on NOD1 expression (Supplementary Fig. S4c). However, we found that the impaired expression of phosphorylated and unphosphorylated Akt could be rescued by CD44a overexpression in NOD1-1IS−/− larvae. Collectively, these findings suggest that NOD1 acts as an activator upstream of the CD44a-mediated PI3K-Akt signaling pathway. Since we can’t find a direct interaction between zebrafish NOD1 and CD44a, the intermediate signaling molecule linking NOD1 and CD44a-mediated PI3K-Akt signaling pathways will be the interest of future study.

In mammals, the phosphoinositide-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is emerging as a critical integrator of intracellular signals, and important for apoptosis, malignant transformation, tumor progression, metastasis and radioreistance40-42. Due to the important role of the PI3K/Akt/mTOR pathway in growth control and cancer research43-45, many valuable inhibitors targeting in this pathway have been developed in recent years46-50. NLRC3 (also known as CLR16.2 or NOD3), another member of the NLR Family, is recently identified as an inhibitory sensor of the PI3K/Akt/mTOR pathway, mediating protection against tumorigenesis in colorectal cancer51. In the present study, the finding that NOD1 is involved in the regulation of NLRC3 homologues, nutrient metabolism as well as the CD44a-mediated PI3K/Akt/mTOR pathway is very intriguing, which suggest the multiple functions of NOD1 in different field of biology including infectious disease, metabolism, oncology, developmental biology, and not confined to the immunology.

In conclusion, the current study revealed the regulation of innate and adaptive immunity by NOD1 during zebrafish larval development. Based on the experimental data mentioned or discussed above and previous reports, a working model of how NOD1 affects intracellular signaling is shown in Fig. 7b. Most notably, the current study demonstrates that NOD1 acts as an activator upstream of the CD44a-mediated PI3K-Akt signaling pathway in larval survival. Future studies will focus on the mechanism by which NOD1 transactivates MHC class I and II related genes, and the effect of the correlation between NOD1 and CD44 signaling in pathogen infection.

**Methods**

**Zebrafish care and maintenance.** NOD1-1IS−/− zebrafish were obtained from the China Zebrafish Resource Center (CZRC). Wild-type AB/TU and mutant zebrafish were raised and maintained under standard conditions52. Zebrafish embryos were obtained by artificial insemination and reared at 28°C. All animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and were approved by the Institute of Hydrobiology, Chinese Academy of Sciences (Approval ID: IHB 2013724).

**Cell culture and transfection.** Epithelioma papulosum cyprini (EPC) and zebrafish ZF4 (embryonic fibroblast cell line) cells were maintained in M199 and DMEM/F12 (1:1) medium supplemented with 10% FBS at 28°C, respectively. Transfection of plasmids was performed using Lipofectamine 2020 (Invitrogen).

**Zebrafish embryo-larval assay.** For the analysis of zebrafish hatching process, the fertilized eggs from the WT and NOD1-1IS−/− parent were randomly divided into four experimental groups, each with 50 embryos. Water was renewed every day in dishes. The hatched larvae were recorded at 2, 3 and 4 dpf to evaluate the hatching rate. For embryo survival analysis, the fertilized eggs from the WT and NOD1-1IS−/− parent were randomly divided into four experimental groups, each with 50 embryos. The dead embryos or larvae were recorded daily until 5 dpf.

For larval survival analysis, the larvae from the WT and NOD1-1IS−/− parent at 4 dpf were randomly divided into four groups, each with 49–52 larvae. Larvae survivals were recorded daily until 9 dph (12 dpf). Dead individuals were removed each day.

**cDNA library construction and Illumina deep sequencing.** Total RNA was isolated from the WT and NOD1-1IS−/− zebrafish at 10 dpf using the TRIzol® Reagent (Invitrogen). The quantity and quality of total RNA were checked by Nanodrop2000 (Thermo Scientific) and Bioanalyzer 2100 (Agilent). Sequencing libraries for whole transcriptome analysis were generated using Truseq™ RNA sample prep Kit (Illumina). After quantification using TBS380 Picogreen (Invitrogen), the libraries were sequenced using Hiseq4000 Truseq SBS Kit V3-HS(200cycles)(Illumina). To identify differentially expressed genes between the WT and NOD1-1IS−/− zebrafish, the expression levels were measured by using numbers of fragments per kilobase of transcript per million fragments sequenced (FPKM). The raw sequences were deposited at NCBI Sequence Read Archive (Accession No. SRP090717).

**PCR arrays and qRT-PCR.** RNA used for PCR arrays was extracted from the WT and NOD1-1IS−/− zebrafish at 10 dpf using the TRIzol® Reagent (Invitrogen). The expression profile of genes involved in various cellular pathways, pathway-specific PCR arrays including zebrafish PI3K-Akt Signaling Pathway RT2 Profiler PCR Array (Qiagen), zebrafish mTOR Signaling RT2 Profiler™ PCR Array (Qiagen) and custom RT2 Profiler PCR arrays (SA Bioscience) containing 372 innate and adaptive immune genes were used. Data was analyzed using the manufacturer’s software. Three biological
replicates were performed for the WT and NOD1-1IS−/− zebrafish. PCR arrays data were deposited at www.ncbi.nlm.nih.gov/geo with the accession numbers GSE80621 and GSE80622. For understanding the expression changes of CD44a, MHC class I and II genes during embryonic and larval development from NOD1-1IS−/− zebrafish, the effect of CD44a overexpression on the expression of NOD1, CD44a, MHC class I and II genes from WT zebrafish, and whether CD44a rescued the expression of MHC class I and II genes from NOD1-1IS−/− zebrafish, RNA was extracted using Trizol (Invitrogen) from snap-frozen embryos or larvae collected at different time points. CDNA was obtained using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) with a oligo(dT)18 primer. QRT-PCR was then performed on a BIO-RAD CFX96 Real-Time System using iQ™ SYBR® Green Mix (BIO-RAD) according to manufacturer’s instructions. Quantifications were performed on triplicate wells. To normalize CDNA amounts, the housekeeping gene GAPDH was used. The primers specific for the gene of interest were listed in Supplementary Table S2.

**Western Blot analysis.** To detect NOD1 antibody specificity, 1 × 10⁶ EPC cells overexpressing NOD1 and NOD2 were lysed in 200 μl cold Pierce® IP lysis buffer (Prod# 87788) containing protease inhibitor (Prod# 1860932), then separated by SDS–PAGE, transferred to PVDF membranes and immunoblotted with the primary antibodies of monoclonal mouse anti-FLAG tag antibody (Sigma, F3165) and monoclonal mouse anti-NOD1 antibody at a dilution of 1:5000 and 1:1000, respectively. To detect endogenous NOD1 protein, 5 × 10⁶ ZF4 cells were lysed in 300 μl cold Pierce® IP lysis buffer containing protease inhibitor (Sigma). For NOD1 expression in WT and NOD1-1IS−/− zebrafish, 50 larvae at 10 dpf were lysed in 200 μl cold Pierce® RIPA buffer (Prod# 89900) containing protease inhibitor using ultrasonication.

For detecting the protein expression involved in PI3K–Akt pathway, 60–250 larvae at 5, 7 and/or 10 dpf from WT and NOD1-1IS−/− zebrafish with or without the transfection of FLAG or CD44-FLAG were lysed in 200–800 μl cold RIPA buffer containing phosphatase inhibitor (Prod #78420) and protease inhibitor using ultrasonication. Primary antibodies used were phospho-Akt (Cell Signaling #4060 S), Akt (Cell Signaling #9227), phospho-GSK-3β (Cell Signaling #9323), GSK-3β (Cell Signaling #9315), phospho-S6 ribosomal protein (Cell Signaling #2215), 6S ribosomal protein (Cell Signaling #2217) and phospho-CREB (Cell Signaling #9198) at a dilution of 1:1000. Mouse monoclonal anti-GAPDH (proteitech, 60004-1-Ig) was used throughout as a loading control. Secondary antibodies were diluted 1:5000 including Pierce goat anti-rabbit IgG and goat anti-mouse IgG (Prod #31460 and #31430). The bands were detected using Pierce ECL Western Blotting Substrate (Prod # 32106) and ECLWestern blot system (LAS-4000mini, Fuji, Japan) according to the manufacturer’s instructions. Densitometer analysis was performed using Quantity One software (BioRad).

**Rescue experiments.** In the rescue experiments, 2-cell embryos from WT and NOD1-1IS−/− zebrafish were microinjected with FLAG or CD44-FLAG plasmid, which were diluted to the desired concentration of 100 ng/μl. The typical injected volume was 2 nl. The injected embryos were raised at 28°C in fish water.

**Statistical analysis.** Expression data by qRT-PCR and western blotting are presented as means and standard error of mean (SEM). Two-tailed Student’s t-test was used to compare statistical significance. All data are representative of two to four independent experiments. The level of significance is shown as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001. Significance testing in the cumulative survival analysis used Log Rank test using GraphPad Prism software.

**Data Availability.** All data generated or analysed during this study are included in this published article and its Supplementary Information files.

**References**

1. Ting, J. P., Duncan, J. A. & Lei, Y. How the noninflammasome NLRs function in the innate immune system. *Science* **327**, 286–290 (2010).
2. Ting, J. P. et al. The NLR gene family: a standard nomenclature. *Immunity* **28**, 285–287 (2008).
3. Schroder, K. & Tschopp, J. The inflammasomes. *Cell* **140**, 821–832 (2010).
4. Bertin, J. et al. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J. Biol. Chem.* **274**, 12955–12958 (1999).
5. Chamaillard, M. et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* **4**, 702–707 (2003).
6. Hasegawa, M. et al. Nucleotide-binding oligomerization domain 1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen. *J. Immunol.* **180**, 4872–4880 (2011).
7. Inohara, N. et al. An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. *J. Biol. Chem.* **275**, 27823–27831 (2000).
8. Girardin, S. E. et al. CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO Rep* **2**, 736–742 (2001).
9. Silva, G. K. et al. Cutting edge: nucleotide-binding oligomerization domain 1-dependent responses account for murine resistance against Trypanosoma cruzi infection. *J. Immunol.* **184**, 1148–1152 (2010).
10. Caruso, R., Warner, N., Inohara, N. & Núñez, G. NOD1 and NOD2: signaling, host defense, and inflammatory disease. *Immunity* **41**, 898–908 (2014).
11. Fritz, J. H. et al. Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity. *Immunity* **26**, 445–459 (2007).
12. Loving, C. L. et al. Nod1/Nod2-mediated recognition plays a critical role in induction of adaptive immunity to anthrax after aerosol exposure. *Infect Immun.* **77**, 4529–4537 (2009).
13. Travassos, L. H. et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat. Immunol.* **11**, 55–62 (2010).
14. Irving, A. T. et al. The immune receptor NOD1 and kinase RIP2 interact with bacterial peptidoglycan on early endosomes to promote autophagy and inflammatory signaling. *Cell Host Microbe* **15**, 623–635 (2014).
15. Schertzer, J. D. et al. NOD1 activators link innate immunity to insulin resistance. *Diabetes* **60**, 2206–2215 (2011).
16. Zhou, Y. J., Zhou, H., Li, Y. & Song, Y. L. NOD1 activation induces innate immune responses and insulin resistance in human adipocytes. Diabetes Metab. 38, 538–543 (2012).

17. Prajapati, B., Jena, P. K., Raiput, P., Purandhar, K. & Seshadri, S. Understanding and modulating the Toll like Receptors (TLRs) and NOD like Receptors (NLRs) cross talk in type 2 diabetes. Curr. Diabetes Rev. 10, 190–200 (2014).

18. Inoue, H. et al. Activation of nod1 signaling induces fetal growth restriction and death through fetal and maternal vasculopathy. J. Immunol. 196, 2779–2787 (2016).

19. Jang, J. H., Kim, H., Kim, Y. I. & Cho, J. H. Molecular cloning and functional analysis of nucleotide-binding oligomerization domain-containing protein 1 in rainbow trout, Oncorhynchus mykiss. Fish Shellfish Immunol. 51, 53–63 (2016).

20. Xie, J., Hodgkinson, J. W., Katzenback, B. A., Kovacevic, N. & Belosevic, M. Characterization of three Nod-like receptors and their role in antimicrobial responses of goldfish (Carassius auratus L.) macrophages to Aeromonas salmonicida and Mycobacterium marinum. Dev. Comp. Immunol. 39, 180–187 (2013).

21. Hou, Q. H. et al. Differential expression analysis of nuclear oligomerization domain proteins NOD1 and NOD2 in orange-spotted grouper (Epinephelus coioides). Fish Shellfish Immunol. 33, 1102–1111 (2012).

22. Park, S. B. et al. Molecular cloning and functional analysis of nucleotide-binding oligomerization domain 1 (NOD1) in olive flounder, Paralichthys olivaceus. Dev. Comp. Immunol. 36, 680–687 (2012).

23. Sha, Z. et al. NOD-like subfamily of the nucleotide-binding domain and leucine-rich repeat containing family receptors and their expression in channel catfish. Dev. Comp. Immunol. 33, 991–998 (2009).

24. Li, M. et al. Expression profiles of NODs in channel catfish (Ictalurus punctatus) after infection with Edwardsiella tarda, Aeromonas hydrophila, Streptococcus iniae and channel catfish hemorrhage reovirus. Fish Shellfish Immunol. 33, 1033–1041 (2012).

25. Swain, B., Basu, M. & Samanta, M. Molecular cloning and characterization of nucleotide binding and oligomerization domain-1 (NOD1) receptor in the Indian Major Carp, rohu (Labeo rohita), and analysis of its inducible expression and down-stream signalling molecules following ligands exposure and Gram-negative bacterial infections. Fish Shellfish Immunol. 32, 899–908 (2012).

26. Chen, W. Q., Xu, Q. Q., Chang, M. X., Nie, P. & Peng, K. M. Molecular characterization and expression analysis of nuclear oligomerization domain proteins NOD1 and NOD2 in grass carp Ctenopharyngodon idella. Fish Shellfish Immunol. 28, 18–29 (2010).

27. Oehler, S. H. et al. The inflammatory bowel disease (IBD) susceptibility genes NOD1 and NOD2 have conserved anti-bacterial roles in zebrafish. Dis. Model. Mech. 4, 832–841 (2011).

28. DeGrendele, H. C., Estess, P. & Siegelman, M. H. Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science 278, 672–675 (1997).

29. Brennan, F. R. et al. CD44 is involved in selective leucocyte extravasation during inflammatory central nervous system disease. Immunology 98, 427–435 (1999).

30. Do, Y., Nagaratti, P. S. & Nagaratti, M. Role of CD44 and hyaluronic acid (HA) in activation of alarcoactive and antigen-specific T cells by bone marrow-derived dendritic cells. J. Immunother. 27, 1–12 (2004).

31. Graham, V. A., Marzo, A. L. & Tough, D. F. A role for CD44 in T cell development and function during direct competition between CD44+ and CD44− cells. Eur. J. Immunol. 37, 925–934 (2007).

32. Steelman, L. S. et al. Contributions of the Raf/MEK/ERK, PI3K/PTEN/Akt/miTOR and Jak/STAT pathways to leukemia. Leukemia 22, 686–707 (2008).

33. Lin, Y. H. & Yang-Yen, H. F. The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. J. Biol. Chem. 276, 40624–40630 (2001).

34. Maisonneuve, C., Bertholet, S., Phlipott, D. I. & De Gregorio, E. Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. Proc. Natl. Acad. Sci. USA 111, 12294–12299 (2014).

35. Abe, T. et al. CD44 participates in Ip10 induction in cells in which hepatitis C virus RNA is replicating, through an interaction with Toll-like receptor 2 and hyaluronan. J. Virol. 86, 6159–6170 (2012).

36. Pourrajab, F., Yazdi, M. B., Zarch, M. B., Zarch, M. B. & Hekmatimoghaddam, S. Cross talk of the first-line defense TLRs with PI3K/Akt pathway, in preconditioning therapeutic approach. Mol. Cell Ther. 6022–6035 (2016).

37. Chang, M. X., Chen, W. Q. & Nie, P. Structure and evolutionary history of a large family of NLR proteins in the zebrafish. Immunity 40, 278–299 (2014).

38. Lewis, K. L., Del Cid, N. & Traver, D. Perspectives on antigen presenting cells in zebrafish. Dev. Comp. Immunol. 40, 85–100 (2012).

39. Stein, C., Caccamo, M., Laird, G. & Leptin, M. Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. Genome Biol. 8, R251 (2007).

40. Faust, I., Nasr, A., Goubran, R. M., Ross, I. M. & Cleary, N. L. Identification of CD44 as a ligand for the vitronectin receptor (VnR) and its role in the mouse. Mol. Cell Biol. 14, 7229–7239 (1994).

41. Howe, K. et al. The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. J. Biol. Chem. 276, 40624–40630 (2001).

42. Lin, Y. H. & Yang-Yen, H. F. The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. J. Biol. Chem. 276, 40624–40630 (2001).

43. Lewis, K. L. & Osburn, D. Jr. NLRP12 Sensor Negatively Regulates Autoinflammatory Disease by Modulating Interleukin-4 Production in T Cells. J Immunol. 190, 6022–6035 (2012).

44. Anand, P. K. & et al. NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. Nature 488, 389–393 (2012).

45. Lukens, J. R. et al. The NLRP12 Sensor Negatively Regulates Autoinflammatory Disease by Modulating Interleukin-4 Production in T Cells. Immunity 42, 654–664 (2015).

46. Zhang, L. et al. NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING. Immunity 40, 329–341 (2014).

47. Cui, J. et al. NLRP3 negatively regulates the NF-kappaB and type I interferon signaling pathways. Cell 141, 483–496 (2010).

48. Allen, I. C. et al. NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRIF-IFN-β signaling pathways. Immunity 34, 854–865 (2011).

49. Tsay, T. B., Chang, C. J., Chen, P. H., Hsu, C. M. & Chen, L. W. Nod2 mutation enhances NF-kappa B activity and bacterial killing activity of macrophages. Inflammation 32, 372–378 (2009).

50. Meissner, T. B. et al. NLR family member NLRs is a transcriptional regulator of MHC class I genes. Proc. Natl. Acad. Sci. USA 107, 13794–13799 (2010).

51. Kobayashi, K. S. & van den Elen, P. J. NLRs: a key regulator of MHC class I-dependent immune responses. Nat. Rev. Immunol. 12, 813–820 (2012).

52. Neetinckx, A., Rodriguez, G. M., Steimle, V. & Kufer, T. A. NLR5 controls basal MHC class I gene expression in an MHC enhancesosome-dependent manner. J. Immunol. 188, 4940–4950 (2012).

53. Cregagh, E. M. & O’Neill, L. A. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol. 27, 352–357 (2006).
58. Becker, C. E. & O’Neill, L. A. Inflammasomes in inflammatory disorders: the role of TLRs and their interactions with NLRs. Semin. Immunopathol. 29, 239–248 (2007).
59. Ramos, H. J. & Gale, M. Jr. RIG-I like receptors and their signaling crosstalk in the regulation of antiviral immunity. Curr. Opin. Virol. 1, 167–176 (2011).
60. Hu, Y. W., Yu, Z. L., Xue, N. N., Nie, P. & Chang, M. X. Expression and protective role of two novel NACHT-containing proteins in pathogen infection. Dev. Comp. Immunol. 46, 323–332 (2014).
61. Wobus, M. et al. CD44 associates with EGFR and erbB2 in metastasizing mammary carcinoma cells. Appl. Immunohistochem. Mol. Morphol. 10, 34–39 (2002).
62. Orian-Rousseau, V. et al. Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin. Mol. Biol. Cell 18, 76–83 (2007).
63. Boursin, L. Y., Singleton, P. A., Zhu, H. & Zhou, B. Hyaluronan promotes signaling interaction between CD44 and the transforming growth factor beta receptor I in metastatic breast tumor cells. J. Biol. Chem. 277, 39703–39712 (2002).
64. Liang, J. et al. CD44 is a negative regulator of acute pulmonary inflammation and lipopolysaccharide-TLR signaling in mouse macrophages. J. Immunol. 178, 2469–2475 (2007).
65. Kawana, H. et al. CD44 suppresses TLR-mediated inflammation. J. Immunol. 180, 4235–4245 (2008).
66. Polivka, J. Jr. & Janku, F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Anticancer Agents Med Chem 14, 150–157 (2014).
67. Guerrero-Zotano, A., Mayer, I. A. & Arteaga, C. L. PI3K/AKT/mTOR: role in breast cancer progression, drug resistance, and treatment. Cancer Metastasis Rev 35, 515–524 (2016).
68. Chang, L. et al. Targeting PI3K/AKT/mTOR signaling pathway in the treatment of prostate cancer radioresistance. Crit. Rev. Oncol. Hematol. 96, 507–517 (2015).
69. Polivka, J. Jr. & Janku, F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Pharmacol. Ther. 142, 164–175 (2014).
70. Schmelzle, T. & Hall, M. N. TOR, a central controller of cell growth. Cell 103, 253–262 (2000).
71. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–293 (2012).
72. Abraham, J. PI3K/AKT/mTOR pathway inhibitors: the ideal combination partners for breast cancer therapies? Expert Rev. Anticancer Ther. 15, 51–68 (2015).
73. Majchrzak, A., Witkowska, M. & Smoliewski, P. Inhibition of the PI3K/Akt/mTOR signaling pathway in diffuse large B-cell lymphoma: current knowledge and clinical significance. Molecules 19, 14304–14315 (2014).
74. Karki, R. et al. NLRC3 is an inhibitory sensor of PI3K-mTOR pathways in cancer. Nature. doi:10.1038/nature20597 (2016).
75. Westerfield, M. The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio) 4th ed, (Univ of Oregon Press (: Eugene, Oregon, 2000).

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Author Contributions
M.X.C. designed experiments. M.X.C., Y.W.H., X.M.W., S.S.R. and L.C. carried out experiments. M.X.C., Y.W.H., X.M.W. analyzed experimental results. M.X.C. wrote and revised the manuscript. P.N. revised the manuscript. M.X.C. and P.N. contributed reagents/materials/analysis tools. All authors reviewed the manuscript.

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