An anti-inflammatory nod-like receptor is required for microglia development

Celia E. Shiau  
*Stanford University*

Kelly R. Monk  
*Washington University School of Medicine in St. Louis*

William Joo  
*Stanford University*

William S. Talbot  
*Stanford University*

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An Anti-inflammatory NOD-like Receptor Is Required for Microglia Development

Celia E. Shiau,1 Kelly R. Monk,1,2 William Joo,1,3 and William S. Talbot1,*

1Department of Developmental Biology, Stanford University, Stanford, CA 94305, USA
2Present address: Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA
3Present address: Neuroscience Program, Department of Biology, Stanford University, Stanford, CA 94305, USA
*Correspondence: william.talbot@stanford.edu
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SUMMARY

Microglia are phagocytic cells that form the basis of the brain’s immune system. They derive from primitive macrophages that migrate into the brain during embryogenesis, but the genetic control of microglial development remains elusive. Starting with a genetic screen in zebrafish, we show that the noncanonical NOD-like receptor (NLR) nlrc3-like is essential for microglial formation. Although most NLRs trigger inflammatory signaling, nlrc3-like acts cell autonomously in microglia precursor cells to suppress unwarranted inflammation in the absence of overt immune challenge. In nlrc3-like mutants, primitive macrophages initiate a systemic inflammatory response with increased proinflammatory cytokines and actively aggregate instead of migrating into the brain to form microglia. NLRC3-like requires both its pyrin and NACHT domains, and it can bind the inflammasome component apoptosis-associated speck-like protein. Our studies suggest that NLRC3-like may regulate the inflammasome and other inflammatory pathways. Together, these results demonstrate that NLRC3-like prevents inappropriate macrophage activation, thereby allowing normal microglial development.

INTRODUCTION

As the only immune cells dedicated to the defense of the CNS, microglia have unique functions and developmental origins (Aguzzi et al., 2013; Ransohoff and Cardona, 2010). microglia are extremely sensitive to signs of infection, injury, and disease of the brain, and they can respond rapidly, depending on the nature of the stimulus (Ransohoff and Perry, 2009; Perry et al., 2010). After infection, for example, microglia engulf pathogens and initiate an immune response, and they can clear cell corpses and promote healing after injury to the CNS. Microglia also interact with and eliminate neuronal synapses, and they may therefore modulate neuronal connectivity (Paolicelli et al., 2011; Peri and Nüsslein-Volhard, 2008; Schafer et al., 2012; Ransohoff and Cardona, 2010). Unlike other CNS cell types, which derive from the neuroectoderm, microglia arise from a subset of primitive macrophages that migrates from the yolk sac into the brain during embryogenesis (Ginhoux et al., 2010, Herbomel et al., 2001). Primitive macrophages from the yolk sac in mouse (Ginhoux et al., 2010) and zebrafish (Herbomel et al., 1999, 2001) enter the brain prior to definitive hematopoiesis and differentiate into microglia that remain in the brain thereafter (Ginhoux et al., 2010). Because microglia in the adult brain derive from the early embryonic macrophage population, early disruption of microglia in the embryo may impair the immune system of the developing and mature CNS. The cellular and molecular mechanisms, however, that mediate the specification, migration, and differentiation of developing microglia remain elusive.

NOD-like receptors (NLRs) are intracellular pattern-recognition receptors that regulate innate immunity and inflammatory processes (Chen et al., 2009; Davis et al., 2011; Mason et al., 2012). They can respond to pathogens and cellular stress by triggering caspase-1-dependent inflammatory signaling or by activating NF-κB to promote production of proinflammatory cytokines (Chen et al., 2009; Davis et al., 2011). Upon recognizing various ligands, putatively through their C-terminal leucine-rich repeats (LRRs) (Chen et al., 2009; Davis et al., 2011; Mason et al., 2012), NLRs form large multiprotein complexes by self-oligomerization at their central NACHT domain. Activated NLRs recruit other proteins through homotypic interactions with their N-terminal domains, typically either a pyrin or a CARD domain, to activate NF-κB through the NODosome pathway or caspase-1 through the inflammasome mechanism (Chen et al., 2009; Davis et al., 2011). Mutations causing aberrant NLR signaling are linked to numerous human inflammatory disorders, such as inflammatory bowel disease, vitiligo, sarcoidosis, and cryopyrin-associated periodic syndromes (Chen et al., 2009; Davis et al., 2011; Mason et al., 2012), underscoring the importance of curbing inflammatory signaling mediated by the NLRs. Recent studies have shown that NLRC3 and NLRP12 can suppress inflammation after challenge by lipopolysaccharide (LPS) or infection (Schneider et al., 2012; Allen et al., 2012; Zaki et al., 2011). Despite the far-reaching roles of the NLRs in controlling innate immunity, the mechanisms that regulate their
functions and maintain homeostasis in the absence of infection are not well understood.

Starting with a genetic screen for mutations that disrupt microglia in zebrafish, we identified a noncanonical NLR, *nlrc3-like*, that is essential for microglia formation in zebrafish. Our analysis demonstrates that *nlrc3-like* prevents runaway inflammation during embryogenesis and thereby allows the development of microglia. In *nlrc3-like* mutants, primitive macrophages adopt an inflammatory phenotype instead of migrating into the brain to differentiate as microglia. In addition, *nlrc3-like* mutants have systemic inflammation, as evidenced by elevation of proinflammatory cytokines and recruitment of neutrophils into the brain and circulation. Transgenic expression of the wild-type *nlrc3-like* gene in macrophages rescued microglia in the mutants, indicating that the gene acts autonomously in macrophages. Our results demonstrate that *nlrc3-like* serves as an essential, cell-autonomous checkpoint in primitive macrophages that prevents unwarranted inflammation in the absence of overt immune challenges and allows the normal development of microglia.

**RESULTS**

*nlrc3-like* Is Essential for Microglia Development

In a genetic screen in zebrafish, we identified *st73* as a recessive mutation that eliminated microglia (Figures 1A and 1B). At 5 days postfertilization (dpf), homozygous *st73* mutant larvae lacked microglial cells (Figures 1A–1D) but did not have any apparent anatomical defects (Figures 1E and 1F). High-resolution meiotic mapping localized the *st73* mutation to a 1.63 Mb region of linkage group 15 (LG15) (Figure 1G). Among the genes in this interval is *nlrc3-like* (LOC100538217) (Figure 1G), which encodes an atypical member of the NLR family that contains the canonical pyrin (PYD) and NACHT domains but lacks the common LRRs thought to be important for ligand sensing and self-inhibition (Davis et al., 2011; Mason et al., 2012). NLRC3-like belongs to an expanded subfamily of teleost NLRs that shares significant similarity to the human NLRC3 (or Nod3), based on sequence comparisons of the NACHT domain (Laing et al., 2008; Hughes, 2006). Sequence analysis identified a nonsense mutation in *nlrc3-like* near the 5’ end of the open reading frame (Figures 1H and 1I). All *st73* mutants analyzed were homozygous for this lesion (n = 300). Injection of synthetic mRNA encoding the wild-type NLRC3-like protein can fully rescue microglia in *st73* mutants and had no effect on wild-type siblings (Figure S1). On the basis of mapping, the identification of the nonsense lesion, and RNA rescue experiments, we conclude that *st73* is a loss-of-function mutation in the *nlrc3-like* gene.

Abnormal Migration and Inflammatory Activation of Macrophages in *nlrc3-like* Mutants

To define the role of *nlrc3-like* in microglia development, we examined different stages of microglia development starting from the formation of primitive macrophages to differentiation of microglia (20–60 hr postfertilization [hpf]) using markers specific to macrophages (*microfibrillar-associated protein 4* [mfap4] and *macrophage-expressed gene 1* [mpeg1]) (Ellett et al., 2011; Zakrzewska et al., 2010) and all leukocytes (*l-plastin*) (Meijer and Spaink, 2011). We detected no difference in macrophage, neutrophil, or overall leukocyte number and distribution, vasculature formation, or blood circulation between mutants and their wild-type siblings at early time points up to 24 hpf (Figure S2; data not shown). By 2 dpf, however, mutant macrophages aggregated in large clusters on the yolk (Figures 2A–2C and S2). Time-lapse imaging revealed that macrophages actively migrated toward and joined these aggregates in the mutant, in clear contrast to wild-type siblings, in which no aggregation occurred (Figure 2C; Movies S1 and S2). Other macrophages in mutants migrated from the yolk, but they often still formed small clusters (Figure 2E, arrowheads). These macrophages...
typically did not enter the brain parenchyma in the mutants but, instead, remained associated with the vasculature (Figure 2D; Movies S3 and S4). Macrophage and microglia markers (mfap4 and apoe, respectively) confirmed the absence of brain macrophages and microglia through 2.5 dpf, even though peripheral macrophages were present (Figure 2E). The formation of aggregates is characteristic of macrophages activated by infection or injury (Renshaw and Trede, 2012). We also observed that macrophages in mutants formed large cytoplasmic vacuoles, often several per cell (Figures 3A–3C), and that a higher fraction of macrophages were TUNEL positive at 2.5 dpf in mutants than in sibling controls (Figure 3D). TUNEL-positive macrophages in the mutants exhibited condensed apoptotic nuclei (Lamkanfi and Dixit, 2010; Lage et al., 2013) or cellular breakdown (Figure 3E), two characteristics of an inflammatory form of apoptosis called pyroptosis (Lamkanfi and Dixit, 2010; Lage et al., 2013). The number of macrophages in the mutants declined significantly by 3–4 dpf (Figure 3F), consistent with an increase in apoptotic macrophages. The active aggregation, vacuolation, and pyroptotic death of macrophages in nlrc3-like mutants resemble phenotypes previously described for macrophages responding to infection (Mujawar et al., 2006; Siracusa et al., 2008; Lage et al., 2013; Renshaw and Trede, 2012), suggesting that nlrc3-like mutant macrophages are activated despite the absence of overt infection. Because macrophage activation is a prominent component of the inflammatory immune response after infection or injury (Mosser and Edwards, 2008), we examined whether the macrophage abnormalities in nlrc3-like mutants were linked to inappropriate inflammation. Using quantitative PCR (qPCR), we found a highly significant increase in all proinflammatory cytokines tested (il-1b, il-8, il-12a, and tnfα) (Rock et al., 2010), but no detectable change in expression of the β-actin control or the anti-inflammatory cytokine il-10 (Moore et al., 2001) in the mutants relative to wild-type siblings (Figure 3G; data not shown). The highly elevated expression of proinflammatory cytokines indicated that macrophages in the mutants were activated in the context of an inflammatory environment. These inappropriately activated embryonic macrophages (microglia precursor cells) do not migrate properly and undergo premature cell death, thus accounting for the lack of microglia in nlrc3-like mutants.
Systemic Inflammation in *nlrc3-like* Mutants

The abnormal activation of macrophages in *nlrc3-like* mutants raised the possibility of a broader dysregulation of the immune system. To investigate whether other immune cells are disrupted in *nlrc3-like* mutants, we examined neutrophils, the only other mature leukocyte present during embryonic stages, before the adaptive immune system is functional (Lam et al., 2004). Whereas neutrophils were excluded from the brain of wild-type embryos, they aberrantly infiltrated the brains of *nlrc3-like* mutants (Figure 4), as demonstrated by examination of the neutrophil markers myeloperoxidase (mPO) and lysozyme C (lyz) (Mejer and Spaink, 2011). In vivo time-lapse imaging of the neutrophil reporter transgene *lyz:EGFP* (Hall et al., 2007) (Figures 4A–4F; Movies S5 and S6) revealed neutrophils freely roaming in the brain of *nlrc3-like* mutants. Quantitation of *lyz:EGFP* neutrophil numbers revealed a significant number of brain neutrophils in *nlrc3-like* mutants, compared with essentially none in wild-type siblings (Figure 4G). Transverse sections confirmed the mislocalization of mutant neutrophils in the brain parenchyma and around vasculature (Figure S3). In addition to brain infiltration, live imaging in *nlrc3-like* mutants also revealed a highly significant increase of neutrophils in circulation (Figures 4H–4J; Movies S7 and S8), starting as early as 1.5 dpf, when primitive macrophages begin to enter the brain in wild-type embryos. Using markers to distinguish neutrophils (*lyz:EGFP* and pan-leukocyte marker L-plastin+) and macrophages (L-plastin+ only), we also found that neutrophils joined the aberrant aggregates of macrophages described above (Figures 2B, 2C, and 4K). The increase of neutrophils in circulation, infiltration of neutrophils into the brain, intermixing of neutrophils and macrophages in abnormal aggregates, and extremely high levels of proinflammatory cytokines collectively indicate systemic inflammation in *nlrc3-like* mutants.

*nlrc3-like* Acts Autonomously in Macrophages to Mediate Normal Microglia Development

In light of such systemic inflammation, we sought to determine whether NLRC3-like acts within macrophages or in other cell types. Using transient Tol2-mediated transgenesis in *nlrc3-like* mutants, we introduced constructs that expressed either the wild-type *nlrc3-like* or control *mCherry* coding sequence in skin, neurons, neutrophils, or macrophages using cell-type-specific regulatory elements (Figures 5A, 5B, and S4). Expression of the wild-type *nlrc3-like* gene in macrophages rescued microglia (>20 microglia/embryo; 24% of the mutants, n = 55), but expression in the other cell types did not (Figure 5). Partial rescue (10–20 microglia/embryo) was also observed in 11% of mutants injected with the macrophage expression construct (Figure 5B). There was one case of partial rescue with the neuronal *nlrc3-like* expression construct (among 25 mutants examined), perhaps due to leaky promoter expression (Figure 5B). No rescue or partial rescue was obtained with any of the control *mCherry* expression constructs (Figures 5A, 5B, and S4). Expression of the wild-type *nlrc3-like* gene in macrophages rescued microglia (>20 microglia/embryo; 24% of the mutants, n = 55), but expression in the other cell types did not (Figure 5). Partial rescue (10–20 microglia/embryo) was also observed in 11% of mutants injected with the macrophage expression construct (Figure 5B). There was one case of partial rescue with the neuronal *nlrc3-like* expression construct (among 25 mutants examined), perhaps due to leaky promoter expression (Figure 5B). No rescue or partial rescue was obtained with any of the control *mCherry* expression constructs (Figure 5B). Driving *nlrc3-like* expression using the myeloid lineage-specific *pu.1*/*spi1*-GAL4 line also rescued microglia in the mutants, providing further evidence that *nlrc3-like* is required in macrophages (Figure S4).
Continues to Be Required after Microglial Progenitors Enter the Brain and Differentiate

To test whether *nlrc3-like* is required after macrophages have entered the brain and differentiated as microglia, we transiently restored wild-type *nlrc3-like* expression by synthetic mRNA injection at the one-cell stage. Injection of *nlrc3-like* mRNA into mutants can restore microglia at 5 dpf and earlier stages (Figure S1; data not shown). By contrast, at 6 dpf, no mutants injected with *nlrc3-like* mRNA exhibited wild-type numbers of microglia (*n* = 45), and more than half of the injected mutants had no microglia detectable by neutral red staining (Figure 5C). Consistent with a continuous requirement in microglia development, *nlrc3-like* mRNA is expressed at every stage we examined in wild-type embryos, from embryogenesis through the early larval period (1–9 dpf; Figure S5). These data indicate that *nlrc3-like* continues to be required to maintain the proper number of microglia at later stages.

To assess inflammation in *nlrc3-like* mutants at later stages, we measured cytokine expression by qPCR. As at 3 dpf (Figure 3G), transcripts for *il-1β*, *il-8*, and *tnfα* were significantly elevated in *nlrc3-like* mutants at 6 dpf, but *il-12α* expression was similar to wild-type siblings (Figure 5D). To determine whether early expression of wild-type *nlrc3-like* is sufficient to suppress inflammation at later stages, we also examined cytokine levels in mutants injected with *nlrc3-like* mRNA. Proinflammatory cytokine expression at 6 dpf was elevated relative to wild-type controls, and the extent of the increase correlated with the number of microglia in the transiently rescued mutants (Figure 5D). In these transient rescue experiments, mutants with more than ten detectable microglia had significantly lower levels of *il-8* and *tnfα* (*p* = 0.01 and 0.03, respectively) than mutants with no microglia. These data indicate that *nlrc3-like* continues to be required to suppress inflammation and allow the development of the full population of microglia, even after microglia progenitors have entered the brain and differentiated.

**Macrophages Are Required for Increased Inflammatory Signaling in *nlrc3-like* Mutants**

The autonomous action of *nlrc3-like* in the macrophage lineage and the integral roles of macrophages in mediating and responding to inflammation (Rock et al., 2010; Mosser and...
Edwards, 2008) prompted us to ask whether macrophages contributed to the systemic inflammation in nlrc3-like mutants. We thus examined proinflammatory cytokine expression after ablating macrophages in nlrc3-like mutants with a morpholino (MO) against interferon regulatory factor-8 (irf8) (Li et al., 2011) (Figure 6A). Macrophage ablation reduced il-1β expression in nlrc3-like mutants to wild-type levels (Figure 6B). In control assays, macrophage ablation in wild-type embryos did not alter il-1β levels, whereas il-1β expression remained elevated in unablated mutants (Figure 6B), consistent with our previous analysis (Figure 3G). All other proinflammatory cytokines (il-8, il12a, and tnfα) examined were also comparable to wild-type levels in macrophage-ablated mutants (Figure 6B). Taken together, these results demonstrate that macrophages are required for the increased inflammatory signaling in nlrc3-like mutants.

### NLRC3-like Requires Both the Pyrin and NACHT Domains and May Interact with the Inflammasome Component ASC

The inflammatory phenotypes of nlrc3-like mutants suggested that this unusual NLR family member might regulate the production of proinflammatory cytokines and induction of pyroptosis by inhibiting the inflammasome (Davis et al., 2011). Previous in vitro studies indicate that cellular and viral proteins with similar domain organizations, containing only the PYD or PYD combined with NACHT, can inhibit the inflammasome through homotypic interactions with other inflammasome components containing these domains (Dorfleutner et al., 2007a, b; Imamura et al., 2010; Stehlik et al., 2003). To examine the possibility that NLRC3-like interacts with components of the inflammasome, we asked whether NLRC3-like protein can bind to the PYD-containing adaptor protein of the
inflammasome, apoptosis-associated speck-like protein (ASC) (Davis et al., 2011). Reciprocal pull-down assays showed that glutathione S-transferase (GST)-tagged zebrafish ASC and maltose-binding protein (MBP)-tagged NLRC3-like can bind (Figure 6C). The unusual pyrin-NACHT structure of NLRC3-like, the systemic inflammation in nlrc3-like mutants, and the potential interaction between the NLRC3-like and ASC proteins suggest that NLRC3-like may negatively regulate inflammasome activity or other pathways mediating inflammatory signaling that involve ASC (Sarkar et al., 2006; Kolly et al., 2008).

To determine which domains may be essential for nlrc3-like function, we performed a structure-function analysis. We designed deletion constructs that express mRNAs encoding NLRC3-like proteins that lack either the NACHT domain (NLRC3-like-ΔNACHT) or the pyrin domain (NLRC3-like-Δpyrin), which are the two recognizable conserved domains in NLRC3-like. Wild-type nlrc3-like mRNA injection can fully rescue microglia in nlrc3-like mutants at 3–5 dpf (Figures 6D and S1), and we assayed the ability of mRNAs encoding the deletion mutants to rescue nlrc3-like mutants (Figure 6D). Both domains are required to rescue nlrc3-like mutants (Figure 6D), providing evidence that the pyrin and NACHT domains are both essential for NLRC3-like function. The requirement for the pyrin domain supports the possibility that NLRC3-like may functionally interact with other pyrin-containing proteins such as ASC (Dorfleutner et al., 2007a; Vajjhala et al., 2012; Liepinsh et al., 2003), and the requirement for the NACHT domain indicates that NLRC3-like may also interact with itself or other NLR proteins that regulate inflammatory signaling.

LPS Challenge Compromises Microglia Development in nlrc3-like Heterozygous Embryos

To further test the relationship between systemic immune activation and nlrc3-like function, we injected LPS into the bloodstream of embryos from nlrc3-like-+/− heterozygous intercrosses at 1 and 2 dpf and analyzed microglial development at 3 dpf. LPS injection reduced microglia formation in many of the injected nlrc3-like-+/− heterozygous embryos (54%, n = 24) and affected one of nine injected wild-type siblings (11%) (Figure 7). These data indicate that nlrc3-like-+/− heterozygotes are more susceptible to disruptions of microglia development when exposed to the LPS inflammatory stimulus than wild-type siblings. These results are consistent with the autonomous function of nlrc3-like in the macrophage lineage (Figures 5A and 5B) but also show that extrinsic inflammatory stimuli can disrupt the development of microglia when nlrc3-like function is partially reduced.

DISCUSSION

Macrophages utilize an array of pattern-recognition receptors, including the Toll-like receptors (TLRs) and NLRs, to recognize and respond rapidly to infection, injury, and cellular stress (Chen et al., 2009; Davis et al., 2011; Mosser and Edwards, 2008). The signaling activities of these receptors must be regulated to maintain homeostasis and prevent inappropriate or chronic inflammation. Previous studies show that TLR signaling is attenuated through multiple mechanisms, including production of decoy receptors and inhibitors of downstream signaling complexes (Anwar et al., 2013; Coll and O’Neill, 2010). Much less is known about the negative regulation of NLRs in vivo.
Our data support a model in which NLRC3-like competes with proinflammatory NLRs for ASC binding to set a threshold level of expression of inflammation. These results highlight the importance of regulating the activation of embryonic macrophages to allow normal microglia development, in addition to the more widely recognized need to control activation of adult microglia to prevent chronic or acute inflammation that is common in many neurodegenerative and autoimmune diseases such as Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis (Cunningham, 2013; Amor et al., 2010).

Our results demonstrate that nlrc3-like is an essential negative regulator of macrophage activation and inflammation. Loss of NLRC3-like causes an inflammatory state in macrophages that profoundly alters their normal course of development. Instead of seeding the brain to form microglia, embryonic macrophages in the mutant actively migrate to form aberrant cellular aggregations in the yolk, fail to enter the brain, and eventually undergo inflammatory cell death. Activity of nlrc3-like continues to be required even after microglial progenitors enter the brain and differentiate because transient expression of the wild-type gene is not sufficient for long-term rescue of microglia or suppression of inflammation. These results argue that the proposed mechanism of cellular and viral proteins containing only the pyrin domain, only the NACHT domain, or a pyrin and NACHT, which inhibit the inflammasome through homotypic binding (Dorfleutner et al., 2007a, b; Imamura et al., 2010; Stehlik et al., 2003). Furthermore, similar to these inflammasome inhibitory proteins, NLRC3-like has a distinctive pyrin-NACHT structure that lacks the canonical LRRs thought to be important for self-inhibition (Rosenstiel et al., 2007; Chen et al., 2009), suggesting that it may be a constitutively active inhibitor. Our structure-function analysis indicates that both the pyrin and NACHT domains are required for NLRC3-like function, suggesting that NLRC3-like may interact with other proteins containing one or both of these domains, including the adaptor ASC.

The zebrafish genome contains a large number of NLR genes homologous to mammalian NLRC3 (Laing et al., 2008), and our analysis provides functional insights into this group of zebrafish NLRC3-like genes. Although its NACHT domain is most similar to the mammalian NLRC3 (Laing et al., 2008; Hughes, 2006), the central NACHT domain is the only major region conserved between the zebrafish NLRC3-like and mammalian NLRC3 proteins. Moreover, the zebrafish NLRC3-like protein has only pyrin and NACHT domains, whereas the mammalian NLRC3 has C-terminal LRRs but lacks an N-terminal effector motif. Our biochemical studies suggest that zebrafish NLRC3-like may suppress inflammation by interfering with interactions between ASC and other inflammasome components. The mammalian NLRC3 protein can also suppress inflammation. A recent study shows that NLRC3 reduces TLR signaling and NF-κB activity after LPS exposure through its interaction with TRAF6 (Schneider et al., 2012). It is also possible that zebrafish NLRC3-like regulates NF-κB activation in an inflammasome-independent manner because highly elevated levels of mRNAs for proinflammatory cytokines were detected in nlrc3-like-deficient embryos. Consistent with this possibility, zebrafish NLRC3-like has three predicted TRAF-interaction motifs (following the consensus Pro/Ser/Ala/Thr-X-Gln/Glu-Glu). Thus, NLRC3-like may repress NF-κB activity by interference with TRAF adaptor proteins in the recruitment of NF-κB, similar to the anti-inflammatory action of NLRC3 and NLRP12 after immune stimulation (Schneider et al., 2012; Allen et al., 2012; Zaki et al., 2011). Alternatively, inappropriate activation of the inflammasome may secondarily induce cytokine expression in nlrc3-like mutants.

Another important and intriguing difference between zebrafish nlrc3-like and the mouse NLR genes known to suppress inflammation, including NLRC3 and NLRP12 (Schneider et al., 2012; Allen et al., 2012; Zaki et al., 2011), is that the mouse hyperinflammatory mutant phenotypes are evident only after LPS injection or infection, whereas systemic inflammation is found in nlrc3-like mutant zebrafish in the absence of immune perturbation. Under standard rearing conditions, wild-type and heterozygous siblings show no signs of inflammation, but nlrc3-like mutants display severe, systemic dysregulation of the immune system. This difference reveals that nlrc3-like is an essential suppressor of inflammation at steady state, highlighting the importance of the regulatory mechanism that controls immune activation not only in response to overt immune challenges but also during normal development.
In summary, we identify an essential role for an NLR that acts as a checkpoint on inflammatory processes and permits the development of microglia in zebrafish. Our analysis of nlrc3-like mutants highlights the deleterious effect that inflammatory activation has on embryonic macrophages and their subsequent development into microglia. The negative regulatory function of NLRC3-like may provide important insights into the maintenance of homeostasis and the etiology of autoimmune and inflammatory disorders.

EXPERIMENTAL PROCEDURES

Zebrafish Lines and Embryos
Experimental protocols involving zebrafish were approved by the Stanford University Institutional Animal Care and Use Committee. Embryos from wild-type (TL, AB/TU, and WIK), Tg(fz:EGFP) (Hall et al., 2007), Tg(mpeg1:EGFP) (Ellet et al., 2011), Tg(kdrl:mCherry-CAAX) (Fujita et al., 2011), Tg(piiu:Ga4-UAS-EGFP) (Peri and Nüsslein-Volhard, 2008), and nlrc3-like(z71/z71) strains were raised at 28.5°C and staged as described by Kimmel et al. (1995). Embryos were treated with 0.0003% 1-phenyl-2-thiourea (PTU) in methylene blue embryo water to inhibit pigmentation.

ENU Mutagenesis, Microglia Screen, and Neutral Red Assay
Founder P0 wild-type males were mutagenized with N-ethyl-N-nitrosourea (ENU) and subsequently outcrossed to raise F1 and F2 families for screening as described by Pogoda et al. (2006). A pilot F3 genetic screen was conducted to identify putative mutants with specific defects in microglia (e.g., loss of microglia), but no apparent anatomical morphological defects at 5 dpf. A total of 74 F2 families were screened, which amounted to analysis of 50.7 mutagenized genomes. Two putative microglia mutants were identified, but only st73 was recovered. Microglia were scored in the live larva by a neutral red dye-staining assay (Herbomel et al., 2001) with modifications. For phenotypic analysis, neutral red assay was used at 3 dpf and later stages by incubating larvae in embryo water containing 2.5 μg/ml neutral red at 28.5°C for 2–3 hr, followed by one to two water changes, and then analyzed 0.5–24 hr later using a dissecting microscope.

Genetic Mapping
st73 mutant embryos were phenotypically sorted from wild-type siblings at 4–5 dpf by a lack of neutral red positiva microglia staining in the head. Bulk segregant analysis with 480 simple sequence length polymorphisms (SSLPs) (Talbot and Schier, 1999) identified markers on LG15 flanking the st73 mutation. High-resolution mapping was conducted using additional SSLPs and single-nucleotide polymorphisms linked to the mutation, which were found from sequencing PCR fragments amplified from genomic DNA of mutants and wild-type siblings. Sequencing segments of genes in the critical interval by sequencing PCR fragments amplified from genomic DNA of mutants and nucleotide polymorphisms linked to the mutation, which were found from resolution mapping was conducted using additional SSLPs and single-nucleotide polymorphisms linked to the mutation.

Whole-Mount RNA In Situ Hybridization
In situ hybridization on whole-zebrafish embryos and larvae from 20 hpf to 5 dpf was performed using standard methods as described by Pogoda et al. (2006). Antisense riboprobes were transcribed from the following gene-coding sequences cloned in PCR-Tip-Topo vector: lpl (518 bp); mrfap4 (627 bp) using primer sequences as described by Zakrzewska et al., 2010; i-1-plastin (1,541 bp of lcpo1); and apoE (505 bp of apoE). Other probes were made from vectors encoding mpo (full-length, Open Biosystems clone 6960294) and kdr (fli1) (Thissie et al., 2008).

Whole-Mount Immunostaining and TUNEL Assay
TUNEL assay (in Situ Cell Death Detection Kit, TMR red; Roche) was performed as previously described by Chen et al. (2010). Subsequent immunostaining on whole-zebrafish embryos or larvae was performed using the anti-L-plastin (Redd et al., 2006) at 1:250–500 dilution and anti-GFP (GT859; GeneTex) at 1:500 dilution, followed by DAPI staining.

Time-Lapse and Fluorescent Imaging
For live imaging, embryos were embedded in 1.5% low-melting point agarose on glass slides. For cryosectioning, postfixed embryos were equilibrated to 30% sucrose, embedded in OCT medium, snap frozen in dry ice-ethanol bath, sectioned at 10–12 μm, and subsequently imaged. All time-lapse and fluorescent images, except images for analysis of vacuolation and apoptosis, were taken on a Zeiss LSM 5 Pascal confocal microscope using the 10x (NA 0.30) and 20x (NA 0.75) objectives, and 488 and 543 nm laser lines with bright field. Images of yolk sac macrophages for assessing cellular vaccination and cell death were taken on an upright Zeiss Axio Imager.M2 microscope using the 20x (NA 0.8) and 63x (NA 1.4) plan-apochromatic objectives. See also the Supplemental Experimental Procedures for further information.

Expression Constructs
Full-length nlrc3-like-coding sequence (XM_003200091.1) was cloned from a 2.5 dpf embryonic cDNA pool and directionally inserted into the pCS2+ plasmid at Xhol/XbaI sites to make pCS2-FL-nlrc3-like for mRNA transcription. Truncated forms (pCS2-nlrc3-like-J2yprin and pCS2-nlrc3-like-JNACHT) were made using fusion PCR and also directionally cloned into the pCS2+ plasmid at Xhol/XbaI sites. All transgenic expression constructs contained the Tol2-transposon sequences for genomic integration. Primers used in cloning are listed in Table S1. See also Supplemental Experimental Procedures for further information.

mRNA, Plasmid, and MO Injections
pCS2-FL-nlrc3-like and truncated forms (pCS2-nlrc3-like-J2yprin and pCS2-nlrc3-like-JNACHT) were transcribed using the Sp6 mMessage machine (Ambion) to produce 5’ capped mRNAs with a poly-A tail. A range of 50–200 pg of nlrc3-like mRNA was injected into one- to four-cell stage embryos; all amounts of RNA tested in this range can rescue microglia in nlrc3-like mutants. Structure-function analysis was conducted using injections of 150–200 pg of mRNA in one- to two-cell stage embryos. Transgenes were transiently expressed by coinjecting α–25 pg of Tol2 plasmid as described above and ~50–100 pg of Tol2 transposase mRNA at one-cell stage. A splice-blocking MO against irf8 was synthesized by Gene Tools and was injected at 2.5–5 ng into one-cell stage embryos as previously described by Li et al. (2011). As negative controls, embryos were injected with water or not injected.

RNA Extraction, RT-PCR, and qPCR
Total RNA was extracted from individual embryos using the RNAqueous-Micro RNA Isolation Kit (Ambion). Embryos were presorted by neutral red phenotype prior to lysis and genotyped using a PCR-restriction digest assay as described above. cDNA was made using oligo-dT primer and SuperScript II or III Reverse Transcriptase (Invitrogen). qPCR was performed on the ABI 7300 Real-Time PCR System or the Bio-Rad CFX384 Real-Time PCR Detection System with SYBR GreenER qPCR reagent (Invitrogen). Additional information is provided in the Supplemental Experimental Procedures.

Protein-Interaction Pull-Down Assay
DNA constructs were made using modified Gateway vectors (Meireles et al., 2009). GST-ASC fusion protein containing the full-length zebrafish ASC sequence (NM_131495.2) was made in pGEX-4T-1 vector. Full-length NLRC3-like protein (XP_003200139.1) was fused to MBP using the vector pMAL-c2X. All proteins were expressed in Escherichia coli BL21 cells (Invitrogen) using IPTG induction. See also the Supplemental Experimental Procedures for further information.

LPS Microinjection
A total of 3–5 nl of LPS from Pseudomonas aeruginosa 10 (Sigma-Aldrich) at 5 mg/ml in PBS with rhodamine dextran (10,000 MW at 1:5 dilution) was injected into the circulation valley (duct of Cuvier) as described by Milligan-Moyle et al. (2011). Embryos were injected at 22–24 hpf and at 48 hpf;
detection of the fluorescent dextran throughout vasculature was used to select for successful injections for later analysis. Injected embryos and control uninjected siblings were analyzed at 3 dpf using the neutral red assay for microglia.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, one table, and eight movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.004.

AUTHOR CONTRIBUTIONS

C.E.S. recovered and mapped the st73 mutation, and conducted all experiments. K.R.M., W.J., and W.S.T. performed the genetic screen. C.E.S. and W.S.T. analyzed the data and wrote the manuscript.

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