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The zebrafish activating immune receptor Nitr9 signals via Dap12

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Abstract

Both inhibitory and activating forms of natural killer (NK) cell receptors are found in mammals. The activating receptors play a direct role in the recognition of virally infected or transformed cells and transduce activating signals into the cell by partnering with an adaptor protein, which contains a cytoplasmic activation motif. Activating NK receptors encoded by the mammalian leukocyte receptor complex (e.g., killer cell immunoglobulin-like receptors) and the natural killer complex (e.g., Ly49s) partner with the adaptor protein DAP12, whereas NK receptors encoded in the CD94/NKG2 complex partner with the adaptor protein DAP10. Novel immune-type receptors (NITRs) found in bony fish share several common features with immunoglobulin-type NK receptors. Nitr9 is a putative activating receptor in zebrafish that induces cytotoxicity within the context of human NK cells. One isoform of Nitr9, Nitr9L, is shown here to preferentially partner with a zebrafish ortholog of Dap12. Cross-linking the Nitr9L–Dap12 complex results in activation of the phosphatidylinositol 3-kinase→AKT→extracellular signal-regulated kinase pathway suggesting that the DAP12-based activating pathway is conserved between bony fish and mammals.

Keywords

Natural cytotoxicity; DAP10; ERK; AKT; DAP12; NITR

Introduction

The cells of the mammalian immune system rely on an intricate network of signaling pathways to differentiate between “self” and “nonself.” The activation or inhibition of these signaling pathways relies on specific cell-surface receptors that engage specific ligands. Receptors can be classified as inhibitory or activating based on the functional outcome of ligand recognition. Natural killer (NK) cells represent a particularly well-characterized example of a cell lineage that utilizes this mode of regulation. When an activating NK cell receptor binds its ligand, the NK cell is activated to kill the target cell; in contrast, when an inhibitory NK receptor binds its ligand, NK cell-mediated killing is repressed.

Primates and rodents utilize different families of NK receptors to effect NK function. Primates typically utilize the killer cell immunoglobulin (Ig)-like receptor (KIR) family of Ig-type receptors, whereas rodents utilize the Ly49 family of lectin-type receptors (Bashirova et al. 2006; Ortaldo and Young 2005; Trowsdale et al. 2001). Cytoplasmic signaling utilized by these disparate receptors is well conserved (Billadeau and Leibson 2002; Cerwenka and Lanier 2000; Djeu et al. 2002). To initiate cell signaling, activating NK receptors physically associate through a positive charge in their transmembrane domain with a negative charge in the transmembrane domain of an adaptor protein. Adaptor proteins

possess cytoplasmic signaling motifs necessary for activating a cellular response. The majority of mammalian-activating NK receptors (KIRs and Ly49s) utilize the adaptor protein, DAP12. In contrast, NKG2D, a lectin-type-activating NK receptor, utilizes DAP10 (Hyka-Nouspikel and Phillips 2006; Takaki et al. 2006), indicating a certain degree of specificity in the transmembrane interactions.

Considering the recent and rapid evolution of mammalian NK receptors (Hao and Nei 2005; Parham 2005; Sambrook et al. 2005), it is reasonable to expect that the functionally equivalent receptors in nonmammalian vertebrate species will be structurally divergent from the mammalian NK receptors. In this regard, novel immune-type receptors (NITRs) are members of diversified multigene families of activating/inhibitory receptors. Their overall organization is somewhat similar to KIRs; however, NITRs consist of either one or two ectodomains. All NITRs possess one N-terminal ectodomain of the IgV type, which may contain a J sequence, and many NITRs possess a second C-terminal ectodomain of the Ig-intermediate (I) type. Thus far, NITR genes have been identified only in bony fish and have been characterized most extensively in zebrafish (Evenhuis et al. 2007; Hawke et al. 2001; Piyaviriyakul et al. 2007; Strong et al. 1999; Yoder et al. 2001; Yoder et al. 2002; Yoder et al. 2004). Approximately 36 different NITR genes, which encompass 12 different families of receptors, are located within the NITR gene cluster on chromosome 7 in zebrafish (Litman et al. 2001; Yoder et al. 2001; Yoder et al. 2004); two additional families are located on a different chromosome (Yoder and Litman, unpublished observations). Although most of the NITRs in zebrafish possess cytoplasmic immunoreceptor tyrosine-based inhibition motifs and are predicted to exhibit inhibitory functions, a single gene, *nitr9*, encodes a positively charged residue within the transmembrane domain and is predicted to encode an activating receptor (Yoder et al. 2004). Nitr9 is expressed in multiple isoforms, all of which include the charged transmembrane residue and are likely to partner with adaptor proteins such as DAP12 or DAP10. Recently, the zebrafish orthologs of *DAP12* and *DAP10* have been identified (Yoder et al. 2007) permitting the evaluation of their roles in Nitr9 signaling.

We have shown that a recombinant form of zebrafish Nitr9, when cross-linked, can induce an activating signal within the context of mammalian NK cells (Yoder et al. 2004). To elucidate the function of Nitr9, a recombinant construct of the Nitr9 isoform, Nitr9L, was transfected into cultured human cells and shown to preferentially associate with epitope-tagged zebrafish Dap12. After antibody-induced cross-linking of the Nitr9L-Dap12 complex, phosphorylation of extracellular signal-regulated kinase (ERK) and AKT is increased. These data suggest that the Dap12 cytoplasmic signaling pathway is a common feature of activating receptor function that is conserved between bony fish and mammals.

Materials and methods

Reverse transcriptase–polymerase chain reaction

Kidneys from adult zebrafish were dissected, and myeloid and lymphoid cell lineages were purified cytometrically based on forward and side scatter and complementary deoxyribonucleic acids (cDNAs) generated as described (Yoder et al. 2007). One microliter of cDNA was subjected to thermal cycling with gene-specific primers and Titanium *Taq* DNA polymerase (BD Biosciences, San Jose, CA) in a 20- μ l polymerase chain reaction (PCR) reaction, and 10 μ l was analyzed by agarose gel electrophoresis. PCR cycles used for detecting *nitr9*, *dap10*, and *dap12*, and *actin* transcripts were: 40 (annealing at 65°C), 30 (annealing at 65°C), 40 (annealing at 65°C), and 25 (annealing at 65°C), respectively. Myeloperoxidase transcripts were detected using 25 PCR cycles (annealing at 70°C), and T cell receptor (TCR) α transcripts were detected using 40 PCR cycles (annealing at 60°C).

Forward and reverse primers span at least one intron and are listed in Table 1. The identity of amplicons was confirmed by subcloning and DNA sequencing.

Epitope-tagged expression constructs

The Myc-tagged Nitr9L expression vector was constructed with the pLM plasmid (a derivative of pcDNA3; Invitrogen, Carlsbad, CA), which incorporates an amino-terminal leader sequence and Myc tag, and FLAG-tagged Dap10 and Dap12 expression vectors were constructed with the pLF plasmid (which incorporates a FLAG tag but is otherwise identical to pLM). Epitope-tagged constructs were generated by PCR amplifying the coding sequence of zebrafish *nitr9L*, *dap10*, and *dap12* (excluding the leader sequence) with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) employing blunt forward primers and reverse primers that include the endogenous stop codon and incorporate a 3' *NotI* site (Table 1). Amplicons were digested with *NotI* and cloned into the *EcoRV/NotI* sites of pLM or pLF and confirmed by sequencing.

Site-directed mutagenesis

The plasmid encoding FLAG-tagged Dap12 was mutagenized using the QuickChange® Site Directed Mutagenesis Kit as recommended by the manufacturer (Stratagene). In brief, residue Asp-42 of Dap12 (GenBank EF158446) was mutagenized to a Val in the FLAG-Dap12 expression plasmid using the ZFDAP12_D→V-top and ZFDAP12_D→V-bot primers resulting in a FLAG-Dap12^{D42V} construct. Residue Tyr-96 of Dap12 (GenBank EF158446) was mutagenized to an Ala in the FLAG-Dap12 expression plasmid using the ZFDAP12_Y→A-top and ZFDAP12_Y→A-bot primers resulting in a FLAG-Dap12^{Y96A} construct. Mutations were confirmed by sequencing. Primer sequences are listed in Table 1.

Cell culture and transfections

The human AD-293 cell line (a derivative of the HEK293 cell line, with improved cell adherence) was obtained from the American Type Culture Collection (Manassas, VA) and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. Approximately 1×10^6 cells were seeded into 100-mm dishes ~24 h before transfections. Cells were transfected with 10 µg of recombinant plasmid DNA as indicated in "Results and discussion" using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The cells were harvested 48 h after transfection and used for further experiments.

Receptor cross-linking

Transfected cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and incubated with 1 µg/ml of anti-Myc antibody (9B11; Cell Signaling Technology, Danvers, MA) on ice for 30 min. After washing with DPBS, cells were incubated with 0.5 µl/ml of affinity purified rabbit anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch, West Grove, PA) at 37°C for 10 min. After washing with DPBS, 1 ml of lysis buffer (1% NP-40, 10 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 50 mM sodium fluoride, 1 mM ethylenediamine tetraacetic acid, 1 mM sodium orthovanadate, 0.25% sodium deoxycholate, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin, and 10 µl each of protease cocktail I and cocktail II [Sigma-Aldrich, St. Louis, MO]) was added to each 100-mm dish, followed by a 30-min incubation at 4°C. Cells were scraped off the plates and centrifuged for 15 min at 12,000×g and 4°C.

Immunoprecipitation and Western analyses

Transfected cells were washed twice with cold 1× DPBS and placed on ice for 30 min in lysis buffer. Cell lysates were centrifuged at $12,000 \times g$ for 15 min at 4°C to remove nuclei and cell debris. The protein concentrations of the soluble extracts were determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA). The cleared lysates adjusted to contain equivalent amount of protein were incubated with 5 µg of the anti-Myc antibody and rotated overnight at 4°C, after which 30 µl of protein G-agarose beads (Sigma-Aldrich) were added and mixed for 2 h at 4°C. The precipitates were washed three times with lysis buffer, and proteins were eluted in 2× sodium dodecyl sulfate (SDS) sample buffer. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes for Western blotting. Western blots were blocked in PBS–Tween (PBST) containing 4% bovine serum albumin for 1 h and then incubated overnight at 4°C in primary antibodies. The anti-FLAG (M2) antibody was obtained from Sigma-Aldrich; all other antibodies were obtained from Cell Signaling Technology. Blots were washed three times in PBST, followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies in PBST containing 5% nonfat dried milk. The blots were washed three times in PBST, and reaction patterns were visualized using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Piscataway, NJ). In some instances, blots were processed in stripping buffer (Pierce, Rockford, IL) at 37°C for 15 min.

Results and discussion

Zebrafish Nitr9, Dap10, and Dap12 are expressed in lymphocytes

Although the actual associations of adaptors with transmembrane receptors were examined using cell transfection assays, it was essential to first determine if the adaptors and Nitr9 are expressed in the same cell lineage. PCR primers were employed that amplify the entire open reading frame of the previously described long (L) and short (S) transcripts of *nitr9* (*nitr9L* and *nitr9S*, respectively). Unexpectedly, *nitr9L* and a *nitr9* transcript that was shorter than *nitr9S* were amplified from zebrafish lymphocytes (Fig. 1a). Sequencing of this smaller transcript revealed a third splice variant of *nitr9*, encoding Nitr9 “super-short” (Nitr9SS). The entire V domain of Nitr9SS is eliminated by splicing (Fig. 1b and c). It is unknown why transcripts of *nitr9S* were not detected in lymphocytes: It is possible that *nitr9S* is not expressed or expressed at levels much lower than *nitr9L* and *nitr9SS* in lymphocytes or that expression of *nitr9S* may be inducible. A reverse transcriptase-PCR strategy was then used to evaluate the expression of *nitr9*, *dap12*, and *dap10* in purified zebrafish lymphoid and myeloid cells. All three genes were shown to be expressed in lymphocytes and not in the myeloid population (Fig. 1a).

Nitr9L preferentially associates with Dap12

The zebrafish genes encoding Dap10 and Dap12 have recently been described (Yoder et al. 2007). To evaluate the potential partnering of Nitr9L with zebrafish Dap10 and Dap12, a plasmid encoding an epitope (Myc)-tagged Nitr9L was cotransfected into AD-293 cells with a plasmid encoding an epitope (FLAG)-tagged Dap10 or Dap12 (Fig. 2a). Coimmunoprecipitation experiments demonstrated a stable, physical association of the Nitr9–DAP12 combination (Fig. 2b, lane 8); Nitr9L failed to stably associate with Dap10 (Fig. 2b, lane 6). It has been shown that antibody-induced cross-linking of certain activating receptors can enhance the association of the receptor with an adaptor protein and is sufficient to trigger the activating signal transduction pathway (Campbell et al. 1998). As expected, cross-linking Nitr9L (with an anti-Myc antibody) increases the physical association between Nitr9L and Dap12 (Fig. 2b, compare lanes 8 and 9; and Fig. 3a, compare lanes 8 and 9), whereas cross-linking the Nitr9–Dap10 combination did not result

in a detectable receptor–adaptor association (Fig. 2b, lane 7). The association of Nitr9L with Dap12 is specific and enhanced when cross-linked with an antibody.

Elucidating the Nitr9–Dap12 signaling pathway

When activated, mammalian DAP12 signals via SYK tyrosine kinase to engage downstream signaling molecules such as PI3K and ERK (Djeu et al. 2002). PI3K and ERK are rapidly activated in NK cells upon receptor cross-linking and account for redistribution of granules, representing a critical step for NK cell-mediated cytotoxicity. Cross-linking activating receptor/DAP12 complexes results in increased phosphorylation of PI3K and ERK, which can be monitored by Western blot analyses using antiphosphorylated AKT (p-AKT) and ERK (p-ERK) antibodies (AKT lies downstream of PI3K and is used as an indicator for PI3K activation). The cotransfection and cross-linking experiments described above were used to determine if zebrafish Dap12 utilizes the PI3K-ERK pathway. Coexpression and cross-linking of the Nitr9–Dap12 complex results in an increased level of p-AKT and p-ERK (compare lanes 8 and 9 in Fig. 3b and c). Mutated forms of zebrafish Dap12: Dap12^{D42V} and Dap12^{Y96A} were utilized to determine if the stimulation of this activating pathway specifically relies on Dap12. Substitution of the transmembrane Asp→Val abrogates the capacity of Dap12 to associate with Nitr9L via an intratransmembranous electrostatic interaction whereas the Tyr→Ala abrogates the function of the immunoreceptor tyrosine-based activation motif within Dap12. Although Nitr9L can associate with Dap12^{D42V}, it appears to be weaker (compare lanes 8 and 9 with 10 and 11 in Fig. 3a). Cross-linking of Nitr9L with Dap12^{D42V} fails to result in the phosphorylation of ERK or AKT (compare lanes 8 and 9 with 10 and 11 in Fig. 3b and c). Similarly, Nitr9L associates with Dap12^{Y96A}, but the cross-linking of this complex also fails to result in the phosphorylation of ERK or AKT (compare lanes 8 and 9 with 12 and 13 in Fig. 3b and c). Taken together, these results suggest that both PI3K and ERK are activated after cross-linking the Nitr9–Dap12 protein complex and indicate that Nitr9L and Dap12 not only physically associate but likely utilize a similar signaling pathway to initiate cytotoxic (e.g., NK) cell-equivalent activation in zebrafish.

It is interesting to note that when the transmembrane domains of activating NITRs are compared to the transmembrane domains of mammalian IgSF receptors, which are known to partner with DAP12, Nitr9L is the only DAP12-associated receptor to possess an Arg (rather than a Lys) within the transmembrane domain (Table 2): The biological significance of this observation remains to be determined.

Concluding remarks

These data demonstrate that *nitr9*, *dap12*, and *dap10* are expressed by zebrafish lymphocytes and strongly suggest that Nitr9L partners with Dap12 *in vivo* in zebrafish lymphocytes. In addition, Nitr9S and the newly identified Nitr9 variant, Nitr9SS, potentially partner with Dap12 as they all possess an identical transmembrane domain. Based on its overall structure, patterns of diversification, and other data presented here, it is reasonable to hypothesize that Nitr9 functions in zebrafish NK cells as well as cytotoxic T cells. In catfish, multiple NITRs are expressed in clonal lines of NK-like as well as cytotoxic T and macrophage cells (Evenhuis et al. 2007; Hawke et al. 2001). A single inhibitory NITR (poNITR1), cloned from Japanese flounder, was found recently to be expressed in T cells (TCRα⁺) as well as B cells (IgM⁺; Piyaviriyakul et al. 2007); however, transcripts of only one catfish NITR gene have been detected in clonal B cell lines (Evenhuis et al. 2007). In humans, KIR receptors are expressed in NK and T cell lineages, whereas other genes encoded in the leukocyte receptor complex (e.g., LILR/LIR/ILT) are expressed in various hematopoietic lineages (Nakajima et al. 2003; Xu et al. 2005). Ongoing efforts are directed at further characterizing the hematopoietic cell types (*in vivo*) that express and/or rely on the function of NITRs as

well as Dap12 and Dap10. It is possible that NITRs as well NK receptors exhibit differential expression and effect alternative functions in different cell lineages (Valiante et al. 1997).

We propose a model incorporating previous and current data on the function of Nitr9L that parallels what is known about NK receptor-DAP12 signaling in mammals (Fig. 4). The major points of this model are that (1) Nitr9L preferentially partners with Dap12, which is enhanced by receptor cross-linking, (2) cross-linking the Nitr9-Dap12 complex results in activating the PI3K→ERK pathway, and that (3) cross-linking of Nitr9, within the context of an NK cell, can induce cytotoxicity (Yoder et al. 2004). NK function likely has a long phylogenetic history. For these various reasons, we have predicted that NITRs may effect this type of function. As the genomes of bony fish are being solved, it is becoming increasingly more likely that NITRs could function in an NK capacity, which is supported by the observations made here on adaptor associations and signaling.

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Fig. 1.
Detection of adaptor protein expression. **a** RT-PCR was used to detect transcripts of the zebrafish *nitr9*, *dap10*, and *dap12* genes in the zebrafish lymphoid and myeloid lineages. In addition to the *nitr9-long* (*nitr9L*) transcript, an alternatively spliced transcript of *nitr9*, *nitr9-super short* (*nitr9SS*) was detected from lymphocytes. RT-PCR of *mpx* provides a positive control for myeloid cells and RT-PCR of *TCRα* provides a positive control for lymphocytes. β -*Actin* is shown as a positive control for both RNA samples. A negative “no template” control was evaluated in parallel with all primer sets. **b** Visual representations of the three isoforms of Nitr9 are shown: The long and short isoforms were previously

described (Yoder et al. 2004). Immunoglobulin (*V* and *J*) and transmembrane (*TM*) domains are indicated. The positive charge within the TM domain is represented by a *plus sign*, and a cytoplasmic tyrosine (*Y*) is also shown. **c** Sequence analyses of the *nitr9SS* amplicon revealed an open reading frame lacking a V domain. The predicted peptide sequence of Nitr9SS is aligned with the previously described Nitr9L and Nitr9S. Amino acid numbering of the proteins is indicated on the *left*. Leader sequences and transmembrane domains are *boxed* (note that this alignment splits the 23 residue leader sequence of NitrSS into two segments). The arginine in the transmembrane domain is indicated with *white text* on a *black shadow*. Residues which are highly conserved in immunoglobulin domains are indicated above the alignment and numbered according to the international ImMunoGeneTics (IMGT) information system (Lefranc et al. 2005). Cysteines that are conserved in NITR I domains are denoted by an *asterisks above* the alignment (Litman et al. 2001)

a

b

Fig. 2. Nitr9L associates specifically with Dap12. **a** Predicted protein structures of recombinant zebrafish proteins utilized in cotransfection assays. Myc-tagged Nitr9-long (*Nitr9L*) is shown on the *left*, and FLAG-tagged Dap10 and Dap12 are shown on the *right*. Nitr9L encodes two extracellular Ig domains of the variable (V) and intermediate (I) types (Yoder et al. 2004). The cytoplasmic ITAM of Dap12 and the putative Dap10 signaling motif, YMINV, are indicated (Yoder et al. 2007). **b** AD-293 cells were cotransfected with plasmids encoding Myc-tagged Nitr9L and FLAG-tagged Dap10 or Dap12 as indicated (*lanes 3–9*). Cells were then preincubated with an anti-Myc antibody for cross-linking (X-link) before immunoprecipitation. pcDNA3 (*lane 1*) was used as a vector control, and isotype-matched antibody (*lane 2*) was used as a negative control for cross-linking. Immunoprecipitates (anti-

Myc) were separated by SDS-PAGE and subjected to Western analyses using an anti-FLAG antibody (*upper panel*). Whole-cell lysates from the same treatments also were separated by SDS-PAGE and Western analyses completed with an anti-FLAG antibody to show the expression level of Dap10 and Dap12 (*bottom panel*)

Fig. 3. Nitr9L–Dap12 signaling relies on key residues within Dap12. AD-293 cells were transfected with plasmids encoding Myc-tagged Nitr9L (*lanes 6–13*) and FLAG-tagged zebrafish Dap12 (*lanes 3, 8, and 9*) or FLAG-tagged Dap12^{D42V} (*lanes 4, 10, and 11*) or FLAG-tagged Dap12^{Y96A} (*lanes 5, 12, and 13*). Nitr9L was then cross-linked with an anti-Myc antibody (*lanes 9, 11, and 13*), and cells were lysed. **a** Nitr9L was immunoprecipitated (anti-Myc) and Western analyses completed with an anti-FLAG antibody to evaluate the association of Nitr9L with Dap12, Dap12^{D42V}, and Dap12^{Y96A} (*top*). Western analysis also was carried out on the whole-cell lysates to verify expression of the Dap12 proteins (*bottom*). **b** Whole-cell lysates also were subjected to Western analyses to detect phosphorylated ERK (*p-ERK*; *top*) as compared to total ERK (*bottom*) and for **c** phosphorylated AKT (*p-AKT*; *top*) as compared to total AKT (*bottom*). Control cells either were exposed to an isotype matched antibody (*lane 2*) or transfected with the parental plasmid, pcDNA3 (*lane 1*). Note the increase in phosphorylation of ERK and AKT after cross-linking Nitr9L in the presence of Dap12 (compare *lanes 8 and 9*)

Fig. 4.

Model for Nitr9L–Dap12 activation of cytotoxic cells. The NK cell-activating pathway in humans is shown in the context of ligand recognition by the activating NK receptor KIR2DS2. DAP12 forms a dimer via two pairs of extracellular cysteines (*dotted lines*) and associates via complementary charges in the transmembrane domain of the activating NK receptor. Upon cross-linking, LCK/FYN phosphorylates the ITAM within the cytoplasmic tail of DAP12 that initiates the PI3K→ERK signal transduction pathway leading to cytokine release and cytotoxicity. A model for Nitr9L–Dap12 activation of cytotoxic cells parallels this process. Zebrafish Dap12 possesses the requisite cysteines for dimerization as well as the potential to interact with Nitr9L via complementary charges in transmembrane domains (Yoder et al. 2004; Yoder et al. 2007). In this study, it is shown that Nitr9L and Dap12 can physically associate and that cross-linking Nitr9L results in increased phosphorylation of AKT and ERK (Fig. 3). Components of the signaling pathway, which have been shown to be downstream of cross-linking Nitr9L/Dap12, are indicated (*gray background*). Cytoplasmic signaling components predicted to be associated with Nitr9L/Dap12 signaling are listed in *black text*.

Table 1

Oligonucleotide primer sequence

Purpose	Primer name	Primer sequence
RT-PCR Dap10	ZFDAP10-F3	AGGGCTTCTAGTGTTTCTCCTCTC
	ZFDAP10-R3	GCCAAAGATCTTCATGTTTTGCAG
RT-PCR Dap12	ZFDAP12-F3	GGATACTGTTTATAATGTTCCCCG
	ZFDAP12-R3	TTCCGATACTGCTGAAGATCACTG
RT-PCR Nitr9	Nitr9-AUG-For	ATGATCAACTTTTGGATTTTGGACTTTTC
	Nitr9-TAA-Rev	TTACTGCTGGTTAGAAACCGAGTTAATCAT
RT-PCR TCR α	TCR α For	GGAACACAAGTTCATGTTGAAAC
	TCR α Rev	GCTCATCCACGCTTTGAAAGTCA
RT-PCR myeloperoxidase	mpx-For	CCAGAACCAGTGAGCCTGAGACACG
	mpx-Rev	CAGTCTAACCATGGGCAGCGCTGCAC
RT-PCR β -actin	Actin For	GGTATGGAATCTTGCAGTATCCAC
	Actin Rev	ATGGGCCAGACTCATCGTACTCCT
Myc-Nitr9L	Nitr9-for-blunt	CACCCAAATGCACCACCTGTGTTTGTAAAC
	Nitr9-rev-NotI ^a	gactgcggccgcTTACTGCTGGTTAGAAAC
FLAG-DAP10	ZFDAP10-For-bluntYSCF	TACTCATGTTTTGGAGTGAGC
	ZFDAP10-rev-NotI ^a	acgtgcggccgcTCATGTTTTGCAGTTTGC
FLAG-DAP12	ZFDAP12-For-blunt	AATCAAGACTGCAGTTCCTGTTACC
	ZFDAP12-Rev-NotI ^a	gcatgcggccgcTTATTTCCGATACTGCTGAAG
FLAG-Dap12 ^{D42V}	ZFDAP12_D→V-top ^b	GGGATCATCACGTGTGTTATTATTCTCAGCTC
	ZFDAP12_D→V-bot ^b	GAGCGTGAGAATAATAACACACGTGATGATCCC
FLAG-Dap12 ^{Y96A}	ZFDAP12_Y→A-top ^b	GAAGTAGAATCACCTGCCAGGAGCTTTACGGA
	ZFDAP12_Y→A-bot ^b	TCCGTAAAGCTCCTGGCAGGTGATTCTACTTC

^aOverhang (5') sequences are in lower case text and include a *Not*I site.

^bThe mutated codons are italicized.

Table 2

Transmembrane domains of DAP12-associated, Ig-domain receptors

Receptor	Transmembrane domain ¹	Species shown
KIR2DS2	HLHVLIGTSVVKIPFTILLFFLL	Human
NKp44	LVPVFCGLLVAKSLVLSALLVWW	Human
CD300e	HFLLVVLLKLPLLSMLGAVFWV	Human
PILRβ	VALAVAVLKTVILGLLCLLLWW	Human
SIRPβ1	LVALLLGPKLLLVGVSAIYICW	Human
TREM1	VFNIVILLAGGFLSKSLVFSVLF	Human
TREM2	TSILLLLACIFLIKILAASALWA	Human
Siglec-H	VQVAMGEAAIKFLLLGICFFFLS	Mouse
CD300d	YFWVLVSLKFLFLSMLGAVLWV	Mouse
Cd200r3	PSLLSILYVKLAVTVLIVGFAFF	Mouse
Cd300R4	LTILYVKMALLVIILLNVGFAFF	Mouse
TREM3	VIVLTCGFILNKGLVFSVLFVFL	Mouse
Nitr9	LSIARTCLLLLVIVMVIIWYCVY	Zebrafish
IpNITR2 ²	FSIVRSVLLCCVILIMIFYCCS	Channel catfish
IpNITR3 ²	LSVFRVGVLAFLIIIFTMYYNVL	Channel catfish
IpNITR4 ²	LSIIRSAVLLLIIFIICLLCTYIG	Channel catfish
IpNITR10/11 ²	LSIFRVGVLAFLMLIFPIILIVF	Channel catfish

¹Transmembrane domains predicted by SMART software (<http://smart.embl-heidelberg.de/>; Letunic et al. 2006). Charged residues are white text on black.

²Catfish IpNITR2, IpNITR3, IpNITR4, IpNITR10, and IpNITR11 encode transmembrane domains similar to zebrafish Nitr9 (Hawke et al. 2001) and are predicted to associate with DAP12.