Nucleotide binding domain and leucine-rich repeat pyrin domain-containing protein 12: characterization of its binding to hematopoietic cell kinase

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Running title: NlrpNLRP12 binds to HekHCK

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Abstract
Protein-protein interactions are key to defining the function of nucleotide binding domain (NBD) and leucine-rich repeat (LRR), pyrin domain (PYD)-containing family of proteins 12 (NlrpNLRP12). cDNA encoding the human PYD + NBD of NlrpNLRP12 was used as bait in a
yeast two-hybrid screen with a human leukocyte cDNA library as prey. Hematopoiesis cell
kinase (HckHCK), a member of the Sc-SRC family of non-receptor tyrosine kinases, was
among the top hits. The C-terminal 40 amino acids of HckHCK specifically bound to
NlrpNLRP12’s PYD + NBD, but not to that of NlrpNLRP3 and NlrpNLRP8. Amino acids F503,
I506, Q507, L510, and D511 of HckHCK are critical for the binding of HckHCK’s C-terminal
40 amino acids to NlrpNLRP12’s PYD + NBD. Additionally, the C-terminal 30 amino acids of
HckHCK are sufficient to bind to NlrpNLRP12’s PYD + NBD, but not to its PYD alone nor to
its NBD alone. In cell lines that express HckHCK endogenously, it was co-immunoprecipitated
with stably expressed exogenous NlrpNLRP12. Also, NlrpNLRP12 co-immunoprecipitated and
co-localized with HckHCK when both were overexpressed in 293T cells. In addition, in this
overexpression system, steady-state NlrpNLRP12 protein expression levels significantly
decreased when HckHCK was co-expressed. Bioinformatic analysis showed that HckHCK
mRNA co-occurred with NlrpNLRP12 mRNA, but not with other NlrpNLRP proteins in
blood and marrow samples from acute myeloid leukemia (AML) patients. The mRNA of
NlrpNLRP12 is also co-expressed with HckHCK in AML patient samples, and the levels of
mRNA expression of each are correlated. Together these data suggest that NlrpNLRP12,
through its binding to HckHCK, may have an effect on the progression of AML.

Introduction

Pattern recognition receptors (PRR) are the first major line of cellular defense against damage-
associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP)(1).
Among PRR, one class of proteins is the nucleotide-binding domain (NBD) and leucine-rich
repeat (LRR) receptor family (NLR). NBD and LRR PYD protein 12 (NlrpNLRP12) is one
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protein that belongs to the PYD subfamily of the NLR class and is a multi-functional protein (2).

Nlrp12’s functions likely all depend on defined protein-protein interactions. For example, in anti-inflammatory functions of Nlrp12, Nlrp12 interacts with tumor necrosis factor receptor associated factor 3 (TRAF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-inducing kinase (NIK) in the non-canonical NF-κB pathway, when toll-like receptors (TLR) are stimulated with Pam3Cys4 triacylated lipopeptides followed by addition of cluster of differentiation 40 (CD40) (3). Dr. Kanneganti’s group also showed that NLRP12 expression downregulated the Nlrp canonical NF-kB pathway during the Salmonella infection (4,5). NLRP12’s inhibition of the non-canonical NF-κB pathway, thereby effecting an anti-inflammatory function, has been reported in many publications (7-11). For example, in colitis-driven colon cancer, Nlrp12 knock-out (KO) mice experience a higher frequency of death, weigh less, have a shorter colon length, and have more severe disease progression than wild type (WT) mice, all of which may occur through Nlrp12’s inhibition of the non-canonical NF-κB pathway (3,4). In addition, Nlrp12 KO mice have been reported to exhibit higher innate immunity when it is infected by the virus because Nlrp12 inhibits the non-canonical NF-κB pathway; thus, in a KO mice, a more significant increase of NF-κB activity is observed compared to WT mice when it is infected by the virus (6). And in the case of Nlrp12 protecting the diversity of intestinal microbiota (7), thereby preventing obesity (8), it has also been reported that Nlrp12 KO mice display significantly decreased intestinal microbial diversity due to increased innate immunity, resulting from the lack of inhibition of the non-canonical NF-κB pathway.
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In addition, NlrpNLRP12 also interacts with interleukin (IL)-1 receptor-associated kinase 1 (IRAK1) in the canonical NF-κB pathway when TLRs are stimulated, also to inhibit the inflammatory response (9). In contrast, for human studies of NlrpNLRP12-associated autoinflammatory disorders, no protein-protein interactions have been reported (10,11).

Functionally, however, NlrpNlrpLRP12 KO mice had a higher immunological response in the autoimmune model of contact hypersensitivity (12); thus, these mice are more prone to develop contact hypersensitivity. This results most likely from NlrpNlrpLRP12 KO mice having defective migration of their dendritic cells (DC). In addition, Luken et al. demonstrated that NLRP12 regulates the Experimental autoimmune encephalomyelitis (EAE) model via regulating T-cell responses (13). But, in these mouse studies (Lukens et al., 2015), no protein-protein interactions had been reported either (12). And NlrpNLRP12 is protective against Yersinia pestis infection through NlrpNLRP12’s activation of the inflammasome which results in secretion of IL-18 and IL-1β. But, again, no protein-protein interactions of NlrpNLRP12 within inflammasomes had been characterized (14). Also, there is also a study showing that NLRP12 also regulates the c-Jun N-terminal kinase (JNK) pathway in hepatocytes (15). But, again, no protein-protein interactions related to how within the NLRP12 activates JNK pathway had not been showed demonstrated. Thus, there is a further need to find and to characterize previously unreported NlrpNLRP12-binding proteins. Knowing NlrpNLRP12’s protein binding partners may help to gain mechanistic insight into pathways previously characterized as being regulated by NlrpNLRP12. Moreover, identifying and validating potentially novel binding partners of NlrpNLRP12 may suggest novel functions for NlrpNLRP12.
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In humans, NLRP12 is mainly expressed in macrophages, DCs, and neutrophils. In mice, NLRP12 is primarily expressed in DCs and neutrophils (6,12,16). The NLRP12 protein has three domains, from N- to C-terminus: PYD, NBD, and LRR domains (2,17,18). It is generally accepted that the LRR domain, like NLRP1’s LRR domain and NLRP3’s LRR domain, is responsible for sensing ligands or other types of activators of NLRPs in modulating their role in an immunologic response (19-22). However, interestingly, it has been reported that without the LRR domain, NLRP3 still can be activated by triggers of the canonical NF-κB pathway (23). The NBD domain is responsible for self-oligomerization when NLRP is activated, and the PYD domain is responsible for regulating downstream signaling pathways (and thus, it is called the “interaction domain”) (16,23). Here we used a truncated form of NLRP12 (PYD + NBD) to identify possible protein-protein interactions that may regulate signaling pathways in which NLRP12 is involved. In addition, to our knowledge, there are currently no protein-protein interaction studies that have been performed for this truncated form of NLRP12 and may thus represent a new general approach to screening for protein-protein interactions involved in signaling complexes with NLRPs.

A yeast two-hybrid screen was performed to identify potential binding partners of NLRP12’s PYD + NBD. HCK, a member of the Src family of non-receptor tyrosine kinases, was one of the top positive clones (“hits”) among all of the proteins that appeared to interact with NLRP12. Further experiments were done to confirm the novel binding of NLRP12 to HCK and to characterize key structural features of the binding interaction. In addition, co-expression of HCK with NLRP12 resulted in a significant decrease of steady-state NLRP12 protein expression levels. Finally,
together with a bioinformatics analysis included here, a potential interaction between Nlrp12 and Hck may be involved in modulating the role of Hck in the pathogenesis of AML (24-26).

Results

Nlrp12 interacts with Hck in the yeast two-hybrid assay

A human leukocyte cDNA library was used as prey in the screening of binding partners for Nlrp12 using the yeast two-hybrid assay. The basic principle of the yeast two-hybrid assay is that if the cDNA library contains clones that express proteins, or parts thereof, that interact with the co-expressed bait, the Nlrp12 PYD + NBD (Figure 1A) in this case, then the co-transformed yeast clones will grow. The cDNA clones from the library that supported growth would then be isolated and characterize, to identify what proteins interact with Nlrp12’s PYD + NBD. The leukocyte library was used because Nlrp12 is mainly expressed in leukocytes in humans (2,16,27,28), and thus putative binding partners for Nlrp12 are also likely to be expressed there.

The screening resulted in 119 positive clones from the leukocyte cDNA library (“hits”) expressed from 48 distinct genes (Supplementary materials, Supporting information, Table S1) that were subsequently identified as encoding for proteins (or portions thereof) that potentially interact with Nlrp12’s PYD + NBD. The clones with the most hits are listed in Table 1. Among the 119 hits, which included repeated hits of the same genes, 9 were individual clones that encoded fragments of a member of the Sc-SRC family of non-receptor tyrosine kinases, Hck, which was among the highest number of hits for a single gene (Figure 1B and Table 1).
Moreover, of the 9 positive \textit{Hck} clones, 5 were encoded distinct \textit{Hck} fragments (Figure 1C). Full-length \textit{Hck} p61 was also obtained as a hit in another independent yeast two-hybrid screen (data not shown). \textit{Hck} has two isoforms, p59 and P61, in Homo sapiens. P61 and p59 share the same transcript, but p61 uses an uncommon start codon CTG, and p59 uses the common one, ATG (29), to give rise to two distinct transcripts. Endogenously expressed in phagocytes, \textit{Hck} p59 is mainly localized to the plasma membrane, while \textit{Hck} p61 is primarily localized to lysosomes, and both of them are also found in the Golgi apparatus (30). It has been suggested that overexpression of p59 promotes the formation of membrane protrusions such as pseudopodia and overexpression of p61 induces podosome rosettes and triggers actin polymerization around lysosomes (30,31). However, there are no differences between p61 and p59 in the region of \textit{Hck} that appears to bind to \textit{Nlrp12}’s PYD + NBD (see below), the C-terminal part of \textit{Hck}. Thus, detecting a full-length clone of only p61 as a hit, opposed to p59, appeared to be coincidental.

\textbf{In directed screens using the yeast two-hybrid assay, \textit{Nlrp12}’s PYD + NBD domain binding to \textit{Hck} C-terminal 40 amino acids appears to be selective for \textit{Nlrp12}, and \textit{Hck} binding to \textit{Nlrp12} appears to be selective for \textit{Hck}}

Initially, the \textit{Hck} C-terminal 40 amino acids (“\textit{Hck} C1 fragment”) was the shortest \textit{Hck} fragment from a positive clone that interacted with the bait, \textit{Nlrp12}’s PYD + NBD (Figure 2A). \textit{Nlrp3} and \textit{Nlrp8} were chosen based on the phylogenetic trees of the PYD + NBD domains of members of the \textit{Nlrp} family: phylogenetically, \textit{Nlrp3}’s PYD + NBD is the most closely related \textit{Nlrp} PYD + NBD to that of \textit{Nlrp12}, while \textit{Nlrp8}’s PYD + NBD is among the furthest related PYD + NBD to that of \textit{Nlrp12}. 

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(Figure 2B). In Figure 2C, yeast clones grew on the 4 drop out (DO) plates (synthetic dropout (SD) plus amino acids supplements minus tryptophan, leucine, histidine, and adenine (-Trp-Leu-His-Ade) and 4DO + 3-Amino-1,2,4-triazole (3-AT) high stringency plates when the yeast were co-transformed with NlrpNLRP12’s PYD + NBD and HekHCK C1 fragment; and thus, this result is indicative of an interaction between the two constructs. However, yeast clones were not observed on 4DO and 4DO + 3-AT plates when NlrpNLRP3’s PYD + NBD and HekHCK C1 fragment were co-transformed, nor when NlrpNLRP8’s PYD + NBD and HekHCK C1 fragment were co-transformed. These results suggest that the HekHCK C1 fragment binds to NlrpNLRP12’s PYD + NBD, but not to those from either NlrpNLRP3 or NlrpNLRP8, results consistent with a selective interaction between NlrpNLRP12’s PYD + NBD and the HekHCK C1 fragment.

Similarly, yeast clones again grew on the high stringency 4DO and 4DO + 3-AT plates when the HekHCK C1 fragment was co-transformed with NlrpNLRP12’s PYD + NBD. However, clones did not grow on the 4DO and 4DO + 3-AT plates when the C1 fragment of other members of the Sc-SRC family of non-receptor tyrosine kinases were co-transformed with NlrpNLRP12’s PYD + NBD (Figure 2D). This experiment suggests that the interaction of HekHCK C1 fragment with NlrpNLRP12 is selective for HekHCK; the C1 fragment of other members of the Sc-SRC family of non-receptor tyrosine kinases did not bind to NlrpNLRP12’s PYD + NBD (Figure 2D).

In directed screens using the yeast two-hybrid assay, amino acids F503, I506, Q507, L510, and D511 of HekHCK are critical for the binding between NlrpNLRP12’s PYD + NBD and HekHCK C-terminal 40 amino acids.
The HckHCK C1 fragment were divided into four helical regions according to its atomic structure (32) (protein data bank (PDB) accession number: 1AD5): R1, R2, R3, and R4 (designated by us) (Figure 3A). As shown in the sequence alignments in Figure 3B, the primary amino acid sequences of R2, R3, and R4 are very well conserved among members of the ScSRC family of non-receptor tyrosine kinases, while R1 is not. To characterize further the important structural features of HckHCK’s binding to NlrpNLRP12’s PYD + NBD, all of the amino acids comprising each of the R2, R3, and R4 domains were mutated to alanine, but only mutated one section at a time was mutated, and these were denoted as the HckHCK M2 fragment, HckHCK M3 fragment, and HckHCK M4 fragment, respectively. The amino acids comprising the R1 region were mutated to those amino acids in the R1 region of the ScSRC family non-receptor tyrosine kinase LYNyn, which has the closest phylogenetic relationship to HckHCK (Figure 3C). All of the mutated constructs are illustrated in Figure 3D. Yeast still grew on the high stringency 4DO + 3AT plates when NlrpNLRP12’s PYD + NBD was co-transformed with the HckHCK M1 fragment, HckHCK M2 fragment, and HckHCK M4 fragment. But, yeast could not grow when NlrpNLRP12’s PYD + NBD was co-transformed with HckHCK M3 fragment (Figure 3E). These experiments suggested that the R1, R2, and R4 regions are dispensable for binding, while R3 may be important for binding. Further analysis was conducted to identify individual critical amino acids of HckHCK’s R3 region binding to NlrpNLRP12’s PYD + NBD using alanine scanning mutagenesis. However, the intervening sequences between R1, R2, R3, and R4 were not examined for binding.

To identify the key amino acid residues involved in binding of R3 to NlrpNLRP12’s PYD + NBD, each amino acid in R3 was individually mutated to alanine in an alanine scanning
mutagenesis analysis, on the background of the HckHCK C1 fragment. As shown by the yeast
two-hybrid assay, the mutations of F503A, I506A, Q507A, L510A, and D511A abolished the
growth of yeast clones on the high stringency 4DO when these yeast strains were co-transformed
with NlrpNLRP12’s PYD + NBD (Figure 3F). These experiments suggest that these four amino
acids are critical for binding. On the other hand, yeast clones still grew on the high stringency
4DO plates when the HckHCK C1 fragments with the E504A, Y505A, S508A, or V509A
mutations were co-transformed with NlrpNLRP12’s PYD + NBD (Figure 3F). These
experiments suggest that E504A, Y505, S508, and V509 are dispensable for binding of HckHCK
C1 fragment to NlrpNLRP12’s PYD + NBD. Whether E504 and I506 are critical for binding is
unfortunately not clear as the yeast did not grow on the control plates (SD/-Trp-Leu). Overall,
however, these results suggest that the amino acids F503, I506, Q507, L510, and D511 in
HckHCK are critical for the binding of HckHCK C1 fragment to NlrpNLRP12’s PYD + NBD.

On the other hand, YES also has five amino acid residues, are all conserved in all
members of the Src-family of non-receptor tyrosine kinases (Figure 3A), suggesting that, while
they are important for binding, they cannot account for the specificity or selectivity of binding
between NlrpNLRP12 and HckHCK. In addition, these four critical residues, as shown in Figure
3C, appear to be in the same plane along a helix, which may then form an interface that is
important for binding to NlrpNLRP12. However, it is unclear whether the structure of the
isolated HckHCK C-terminus used in the screens here will retain the its structural features that it
possesses within the context of full-length HckHCK, due to the potential loss of
intermolecular interactions for the C-terminal region. Moreover, for example, in addition, the
amino acids R500, P501, T502, D512, T515, Q520, and Y521, are 100% conserved among all
other members of the Sc-SRC family of non-receptor tyrosine kinases and are therefore unlikely to play a role in specificity and selectivity of binding of NlrpNLRP12 with HckHCK. But, it is possible that two or more amino acids in the intervening sections between the R1-R4 regions, which were not analyzed by mutagenesis, may then actually account for the specificity and selectivity of binding of HckHCK to NlrpNLRP12. One example is that the combination of R496, P497, and Q522 is unique to HckHCK compared to other members of the cS-SRC family of non-receptor tyrosine kinases.

In directed screens using the yeast two-hybrid assay, the shortest fragment of HckHCK that binds to NlrpNLRP12’s PYD + NBD is its C-terminal 30 amino acids. The shortest form of HckHCK that bound to NlrpNLRP12’s PYD + NBD was determined by observing whether the yeast clones grew on high stringency 4DO or 4DO +3AT plates when NlrpNLRP12’s PYD + NBD was co-transformed with HckHCK fragments C1 to C7 and N1 to N5 (Figure 4) (data of NlrpNLRP12’s PYD + NBD co-transformed with HckHCK fragment C7 did not shown). HckHCK’s C1 to C7 fragments were gradually truncated from the N-terminus of the HckHCK C-terminal 40 amino acid fragment (Figure 4A and 4B). HckHCK N1 to N5 fragments were gradually truncated from the C-terminus of the HckHCK C-terminal 40 amino acid fragment (Figure 4BC and 4D). Yeast strains grew on high stringency 4DO or 4DO +3AT plates when either the HckHCK C1, C2, or C3 fragment was co-transformed with NlrpNLRP12’s PYD + NBD. Thus, of all of the constructs screened, the shortest fragment of HckHCK that can apparently bind to NlrpNLRP12’s PYD + NBD is the C-terminal 30 amino acids, as indicated by the HckHCK C3 fragment (Figure 4B and 4D). In addition, the last C-terminal 5 amino acids of the HCK C-terminus also appear to be important for binding, as clones co-transformed with
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248 **NLRP12**'s PYD + NBD with *N1*, with a deletion of the C-terminal 5 amino acids of 249 **HCK**, did not grow on the high stringency 4DO and 4DO + 3AT plates (Figure 4BD). The 250 structures of both inactive **HCK** (PDB accession number: 1AD5) and active c-SSRCr (PDB 251 accession number: 1Y57), the prototypical member of this family of nonreceptor tyrosine kinases 252 related to **HCK**, show that the last C-terminal 4 amino acids of c-SSRCr are surface- 253 exposed, indicating that these four amino acids that are critical for binding are available to 254 interact with **NLRP12** (33,34).

**NLRP12 co-immunoprecipitates with HCK from mammalian cells, and**

**NLRP12 is co-localized with HCK in mammalian cells**

256 **NLRP12** and **HCK** could be co-immunoprecipitated when they were both 257 heterologously co-expressed in 293T cells (Figure 5A). Also, semi-endogenous conditions were 258 used for co-immunoprecipitations from cells in which **HCK** is endogenously expressed, and 259 **NLRP12** was heterologously stably expressed in epitope-tagged form, such as in 260 macrophage-like RAW 264.7 cells (Figure 5B), monocyte-like THP-1 cells (Figure 5C), and 261 lymphocyte-like U937 cells (Figure 5D). Semi-endogenous conditions have been used in 262 previous reports where **NLRP12** was shown to interact with TRAF3 (3), NIK (35), and 263 IRAK-1(9). In all cases, **NLRP12** co-immunoprecipitated with the p59 and p61 isoforms of 264 **HCK**, but from these experiments, it is not clear that whether **NLRP12** interacts with 265 p59, p61, or both isoforms of **HCK**. In U937 cells and THP-1 cells, since heterologous 266 **NLRP12** expression level was low, adding phorbol 12-myristate 13-acetate (PMA) (1 µM 267 for 24 hr) increased **NLRP12** expression level by stimulating the PMA-sensitive 268 cytomegalovirus promoter of **NLRP12**’s expression plasmid (Figure 5C and 5D). Taken
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together, these data suggest that NlrpNLRP12 and HckHCK (either p59, p61, or both isoforms) can interact with each other in a mammalian cell system, a result that in part validates the findings from the yeast two-hybrid assay.

After heterologous co-expression of NlrpNLRP12 with either the p61 or the p59 isoform of HckHCK in 293T cells, immunofluorescent staining of NlrpNLRP12 and HckHCK showed that NlrpNLRP12 can be co-localized with either p61 or p59 isoforms of HckHCK in most of the 293T cells (Figure 5E and Supplementary materials, Supporting Information Figure S1). The co-localization of NlrpNLRP12 and HckHCK is consistent with an intracellular interaction between the two proteins. In addition, the lack of selectivity of NlrpNLRP12 co-localizing with either p61 or p59 isoform of HckHCK is consistent with the results from the screening assay, where the putative binding region of HckHCK to NlrpNLRP12 is identical in both the p61 and p59 isoforms.

The steady-state level of NlrpNLRP12 protein may be significantly decreased when HckHCK protein is co-expressed in an increasing amount.

Figure 6 shows that in 293T cells, the co-expression of increasing amount of HckHCK may led to a significant decrease in steady-state protein expression levels of NlrpNLRP12. Although, alternatively, the apparent decrease in expression level of NlrpNLRP12 might be due actually to a decrease in the epitope-tag itself or a decrease in the expression of the epitope-tagged protein only. Interestingly, HckHCK protein levels did not show a linearly increasing level of expression upon transfection with increasing amounts of plasmid DNA, possibly due to HckHCK’s maximum expression levels in these cells being achieved with the lowest amounts of
plasmid DNA, which may be resolved if the transfections are performed with lower amounts of plasmid DNA. This lack of linearity in protein expression could also be due to saturation of the signal for HCK on the blot at relatively low levels of HCK expression is saturated or a combination of HCK’s maximum expression levels has been reached as well as saturation of the blot, as mentioned above. Alternatively, reduction of NLRP12 expression could be due to plasmid competition; that is, since the expression of both genes from either plasmid are driven from the same promoter, an increase in the amount of one plasmid may competitively decrease expression of the gene from the other plasmid due to their competition for transcription factors.

In blood and marrow samples from patients with AML, \textit{NlrpNLRP12} and \textit{HckHCK} co-occur and are co-expressed. The odds ratio test (a statistical test that defines the likelihood of association of two events) determines whether two proteins either co-occur or are mutually exclusive in their expression. The Fisher exact test is a statistical test that uses P-values to determine if two events, such as co-occurrence or mutual exclusivity, are quantitatively significantly associated. Applying these tests to The Cancer Genome Atlas (TCGA) provisional data sets for AML patients (36-38), \textit{NlrpNLRP12} and \textit{NlrpNLRP1} are the only two \textit{NlrpNLRP} family members that show a statistically significant co-occurrence with \textit{HckHCK} (Table 2). However, \textit{NlrpNLRP1}’s co-occurrence has a P value of 0.048, while \textit{NlrpNLRP12}’s co-occurrence has a P value of <0.001. Therefore, \textit{NlrpNLRP12} is most likely the only \textit{NlrpNLRP} family protein mRNAs that shows co-occurrence with \textit{HckHCK} in samples from patients with AML.
Moreover, in this analysis, Nlrp12 is significantly co-expressed with Hck (Pearson coefficient = 0.80 and Spearman coefficient = 0.73, Pearson and Spearman coefficients show the possibility of a linear relationship of two factors, Figure 7). This analysis is also consistent with Nlrp12 and Hck co-occurring in the same patients with AML. However, Nlrp12 was not the only Nlrp protein that shows significant co-expression with Hck. Nlrp1 and Nlrp3 also showed significant co-expression with Hck. This can be explained by the fact that co-expression is less stringent than co-occurrence. In summary, Nlrp12 is the only Nlrp protein that shows co-occurrence with Hck, and it also shows co-expression with Hck, although it is not the only member of the Nlrp family to do so. Other data sets, including Hausera data, Metzeler 1 data, Metzeler2 data, and Raponi data, were not available to evaluate the co-expression of Nlrp12 versus Hck, as these other data sets did not incorporate data regarding Nlrp12 expression.

Other potential binding partners of Nlrp12 from the yeast two-hybrid screen
Other potentially interesting and unique genes that whose products may interact with Nlrp12’s PYD + NBD were identified in the yeast two-hybrid screen (Table 1 and Supplementary materials, Supporting information Table S1). Table 1 only shows those hits with frequency larger than 2, and these hits were analyzed through an Ingenuity Pathway Analysis (IPA) diagram (Figure 8). In the IPA diagram, protein-protein interactions found by our yeast two-hybrid screen are connected by solid lines and interactions between protein binding partners collated by IPA are connected by dashed lines. In addition, those molecules that are associated with AML are colored purple. Interestingly, Nlrp12 is also associated with AML from the
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Database Catalogue of somatic mutations in cancer (COSMIC). Although the mutation occurs in the NBD of NlrpNLRP12, only 2 samples of these mutations are observed within 175 samples and may be worthy of follow-up study.

The interaction of one of the putative binding partners to NlrpNLRP12 identified in our screen, TRAF3 interacting protein 3 (TRAF3IP3), was validated in a co-immunoprecipitation experiment where the FLAG-tagged form of TRAF3IP3 and V5-tagged form of NlrpNLRP12 were overexpressed in 293T cells. TRAF3IP3 was coimmunoprecipitated by adding mouse monoclonal anti-TRAF3IP3 antibody, and a band that showed the same molecular weight size of NlrpNLRP12 was detected by rabbit monoclonal anti-V5 antibody (Figure 8). However, further validation of these other putative interactors detected by the yeast two-hybrid needs to be done.

Discussion

In this paper, the yeast two-hybrid assay was used to screen for NlrpNLRP12 binding partners. This approach identified 48 genes as positive hits encoding proteins that could possibly bind to NlrpNLRP12. Among the hits, the cS-SRC non-receptor tyrosine kinase family member HeKHCK was selected to focus upon as a potentially interesting target, particularly given its association with hematopoiesis and AML. Further direct screens found that NlrpNLRP12 specifically binds to HeKHCK, but not to NlrpNLRP8 or NlrpNLRP3. Conversely, HeKHCK, but not other members of the SRCc-SRC non-receptor tyrosine kinase family, specifically binds to NlrpNLRP12. HeKHCK’s C-terminal 30 amino acids are sufficient for binding to NlrpNLRP12. And, amino acids F503, I506, Q507, L510, and D511 of HeKHCK are critical for binding to NlrpNLRP12. Co-immunoprecipitation experiments from mammalian cells
confirmed that *NlrpNLRP12* and *HckHCK* interact. Co-expression of *NlrpNLRP12* and *HckHCK* results in a significantly lower steady-state expression level of *NlrpNLRP12*; thus, the interaction may affect *NlrpNLRP12* protein stability. Finally, a bioinformatics analysis shows that *NlrpNLRP12* and *HckHCK* essentially exclusively co-occur, and mRNA levels for both are correlated in blood samples from AML patients. Together, these data suggested that *NlrpNLRP12* and *HckHCK* protein and mRNA co-expression may have an effect on the AML.

The binding data from yeast two-hybrid (Figure 3 and Figure 4) showed that the R2 and R3 regions are both important for binding, but we suspect that R3 region is the major region that is critical for binding, while R2 region may be an “auxiliary” region that is also critical for binding, because when we delete the R2 region but maintain the R3 region, the R3 region itself cannot maintain stable binding of *HckHCK*’s C-terminal 40 amino acids to *NlrpNLRP12*’s PYD + NBD. Thus, binding of *NlrpNLRP12*’s PYD + NBD to this fragment of *HckHCK* may occur initially through binding to R3, followed by stabilization of the interaction by secondarily binding to R2. This model is also consistent with the finding that when we mutated the R3 region, the binding of *HckHCK*’s C-terminal 40 amino acid fragment to the *NlrpNLRP12*’s PYD + NBD was lost despite the R2 region being present. The above evidences are consistent with the model that R2 and R3 regions together are both necessary for binding, but the stabilization of binding to the R3 region may require the presence of R2.

The yeast two-hybrid screen with *NlrpNLRP12* also identified products from 47 other genes as potential interactors. According to the Search Tool for Recurring Instances of Neighbouring Genes (STRING) protein network and previous publications, *NlrpNLRP12* interactors include...
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IRAK1 (39), NIK (35), TRAF3(3), heat shock protein 90 (HSP90) (40), Fas associated factor 1 (FAF1) (41), and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) proteins. IRAK1, NIK, TRAF3, and Heat shock protein (HSP) 90 were only found in experiments involving semi-endogenous protein co-immunoprecipitations with NLRP12. FAF1 was found to be an interacting protein in NMR experiments by showing a difference in the chemical shift perturbation of FAF1’s ubiquitin-associated domains (UBA) domain alone and NLRP12’s PYD domain bound to FAF1’s UBA domain. And FAF1 was also found to interact with NLRP12 in a yeast two-hybrid study where the PYD domain of different NLRP proteins were screened (42). However, in our study, IRAK1 (43), NIK (44), TRAF3 (45), HSP90 (46), FAF1 (47), and ASC (48) proteins were not found in our results of interacting proteins, although they are all expressed in leukocytes. Therefore, given these differences, the results from this yeast two-hybrid screen may provide a unique database for future investigations on the proteins that interact with NLRP12, and the functional outcomes of these interactions.

How HCK co-expression affects NLRP12 expression can be further investigated to determine the functional outcomes of the NLRP12-HCK interaction relative to protein stability or degradation. For example, MG-132 and chloroquine, which are a proteasomal inhibitor and an inhibitor of lysosomal degradation, respectively, can be added to cells in these experiments to determine whether the decreasing protein expression level of NLRP12 in the presence of HCK is due to proteasomal degradation or lysosomal degradation. In addition, an NLRP12 stable cell line, with a typically lower level of NLRP12 expression, compared to transiently transfected cells, can be used to test formally whether NLRP12
protein expression levels are increasingly decreased when the HckHCK’s expression of HCK is increased. Moreover, the rate of Nlrp12 degradation, in the absence or presence of HckHCK, can be studied and compared in pulse-chase assays (35).

Given the interaction that was characterized between Nlrp12 and HckHCK, we sought to identify diseases in which their interaction may be relevant. AML is a type of blood and bone marrow cancer that is characterized by an unusually high level of circulating immature white blood cells (49). HckHCK is found to be highly expressed in hematopoietic stem cells of AML patients (24). The overexpression of HckHCK is linked with the development of AML (24,50). Therefore, HckHCK may be potentially used as a blood biomarker to identify those patients with potential to develop of AML disease. In addition, activation of HckHCK and its signaling pathways often causes the development of AML (25,51). HckHCK has been reported to be involved in the feline McDonough sarcoma (fms)-like tyrosine kinase 3 (FLT3)-HCK-HCK-cyclin D-dependent kinase (CDK6) pathway. The mutation in FLT3-internal tandem duplications (FLT3-ITD) causes the activation of HckHCK, and subsequently results in overexpression of CDK6 in MV4-11 cells, a human AML cell line with the FLT3-ITD mutation. CDK6 siRNA resulted in MV4-11 cells with the FLT3-ITD mutation having a lower proliferation rate compared to MV4-11 control cells (25). Also, it has been reported that in AML patients, HckHCK, LynLYN, and Fgr-FGR phosphorylation levels are high (51). In summary, the above evidence suggests that either high expression and/or high activation of HckHCK could occur in AML patients as well.
Our bioinformatics analysis revealed that Nlrp12 is likely the only member of the NLRP family that shows significant co-occurrence with Hck at the level of mRNA expression. However, our cellular functional study revealed that Nlrp12 protein expression levels decreased when it is co-expressed with Hck. This discrepancy might be due Nlrp12 mRNA expression level not correlating with protein expression level (52-54). Nevertheless, these data are still consistent with the possibility that Nlrp12 may have an effect on AML through its binding to Hck. Nlrp12 and Hck co-expression and co-occurrence may then also be relevant to the prognosis of AML. However, further analyses need to be performed to evaluate the ability of Nlrp12 and Hck to serve as biomarkers in the prognosis of AML.

Nlrp12 and Hck co-expression and co-occurrence may indicate that Nlrp12 and Hck mRNA or protein expression can impact the same pathway or both be regulated by the same molecule. For example, in the case two molecules exhibiting co-occurrence, they may interact in the same signaling pathway, for example, Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and glycogen synthase kinase 3 beta (GSK3β) showed co-occurrence in the AML provisional data, and they are in the same signaling pathway (55). However, further analyses need to be performed to evaluate the ability of Nlrp12 and Hck to serve as biomarkers in the prognosis of AML.

Although many Hck inhibitors have been developed, they often lack specificity, with Hck inhibitors also inhibiting other members of the Sc-Src non-receptor tyrosine kinase family (24,56-5960). If the potential for the development of AML can be decreased when
NLRP12 binds to HCK

NLRP12 does not bind to HCK, then an inhibitor to the interaction between NLRP12 and HCK can be designed. On the other hand, if the potential for AML is increased when NLRP12 and HCK does not bind, then a drug that facilitates NLRP12 and HCK interaction could be developed. By this approach, the non-specificity problem with NLRP inhibitors may still exist but perhaps to a smaller extent, as NLRP proteins are less conserved overall compared to the members of the Sc-SRC non-receptor tyrosine kinase family. For example, the amino acid identity between NLRP12’s PYD + NBD versus another NLRP PYD + NBD most closely related to NLRP12, NLRP3’s PYD + NBD, is 54%. But, the protein identity between the HCK C1 fragment versus the one with the closest relationship, the LYN C1 fragment, is 78%.

In conclusion, this paper reports the preliminary findings of NLRP12 interacting with HCK, and NLRP12 co-occurs with HCK in AML patients. The binding data presented here will expand the database regarding the interactors of NLRP12, and additional bioinformatic and functional in vitro and in vivo analyses may shed light on the functional consequence of the interaction of NLRP12 with HCK.

Material and Methods

Reagents and cells

U937, THP-1, RAW 264.7, and 293T cells were all purchased from American Type Culture Collection (ATCC) (Manassas, VA). pCDNA-NLNP12 plasmid and pCDNA-NLNP3 plasmid were gifts from Dr. Jenny P. Y. Ting (University of North Carolina-Chapel Hill). pCDNA-NLNP8 plasmid was purchased from Genescript (Piscataway, NJ). All other
Chemicals were reagent grade. The mouse monoclonal anti-FLAG antibody and the rabbit monoclonal anti-FLAG antibody were purchased from Sigma-Aldrich (St. Louis, MO). The mouse monoclonal anti-Hck antibody, the mouse monoclonal anti-TRAF3IP3 antibody, and the mouse IgGκ binding protein-horseradish peroxidase (HRP) for detecting the anti-Hck antibody were purchased from Santa Cruz Biotechnology (Dallas, TX). The rabbit monoclonal anti-V5 antibody and polyclonal rabbit HRP-conjugated anti-mouse IgG secondary antibodies were purchased from Cell Signaling (Beverly, MA). The goat anti-rabbit IgG HRP-linked secondary antibody was purchased from Cell Signaling. The Clean-Blot™ IP Detection Reagent was purchased from Thermo Fisher Scientific (Grant Island, NY). Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 488 and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555 were purchased from Thermo Fisher Scientific (Grant Island, NY). The 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from Thermo Fisher Scientific.

Cloning

NLRP12’s PYD + NBD (corresponding to a.a. 1-531) was amplified by polymerase chain reaction (PCR) (primers see Table 3), from a pCDNA-NLRP12 plasmid, was digested with enzymes NdeI and SalI, and subcloned into the pGBK7 vector (Takara). The sequence encoding the PYD + NBD of NLRP3 (corresponding to a.a. 1-536) was amplified by PCR (see table 3 for primers), digested with enzymes NdeI and BamHI, and subcloned into the pGBK7 vector. The sequence encoding the PYD + NBD of NLRP8 (corresponding to a.a. 1-527) was amplified by PCR (see Table 3 for primers), digested with enzymes EcoRI and BamHI, and subcloned into the pGBK7 vector. The entire sequences corresponding to of the
C-terminal 42 amino acids of \textit{FGGr}, \textit{Sc-SRC}r, \textit{YES}es, and \textit{FY}n, and 40 amino acids of \textit{BLK}k, \textit{LCK}k, \textit{LY}n, and \textit{He}k\textit{HCK} (The C terminal 40 (or 42) amino acids is called “C1”), and \textit{He}k\textit{HCK} constructs C2, C3, C4, C5, C6, C7, N1, N2, N3, N4, N5, M1, M2, M3, M4, and \textit{He}k\textit{HCK} C1 mutations for alanine scanning with F503A, E504A, Y505A, I506A, Q507A, S508A, V509A, L510A, and D511A, were all synthesized from IDT DNA (Coralville, IA) and cloned into the pACT2 vector (Takara). \textit{NlrpNLRP12} was amplified by PCR (see Table 3 for primers), digested with AflII and XbaI, and subcloned into pEFIRES-P-3FLAG (601) and pEFIRES -P-V5 (604). 3FLAG and V5 are homemade, i.e., added later into pEFIRES-P. The peptide sequence of the 3FLAG epitope is DYKDDDDKDYKDDDDKDYKDDDDK. The peptide sequence of the V5 epitope is GKIPNPLLGLDST. The multiple cloning sites of pEFIRES-P were modified according to our needs. Sequences encoding \textit{He}k\textit{HCK} p61 and p59 isoforms were amplified by PCR (see Table 3 for primers), digested with AflIII and XbaI, and subcloned into pEFIRES -3FLAG vector. Final plasmids were sequenced to confirm the constructs.

\textbf{Yeast two-hybrid assay}

\textit{Yeast transformed with the plasmid PGBKT7- NlrpNLRP12’s PYD + NBD}

Yeast strain AH109 (Takara, Mountain View, CA) was stored frozen at -80°C. Upon use, it was streaked onto an agar plate supplemented with yeast extract, peptone, dextrose, and adenine hemisulfate (YPDA) (Takara), which was then placed in a 30°C incubator overnight. The growing colonies were picked and placed into 10 ml of YPDA media, incubated overnight with shaking at 30°C. Then they were transformed with \textit{NlrpNLRP12}’s PYD + NBD plasmid, following recommendations in the Takara manual (Matchmaker™ Gal4 Two-hybrid System 3
and Libraries User Manual). A 4 ml of 1 M of 3-AT was added into 800 mL of the YPDA medium that were used for making the plates due to a leakage problem. 3-AT is a competitive inhibitor for the product of the \textit{HIS3} reporter gene. Yeast cells were lysed using a trichloroacetic acid (TCA) method, and western blots were performed to check \textit{NLRP12}’s PYD + NBD protein expression (data not shown).

\textit{Library scale transformation of cDNA library into yeast that have been transformed with the PGBK7- \textit{NLRP12} PYD + NBD plasmid}

A human leukocyte Matchmaker\textsuperscript{TM} cDNA library was purchased from Takara. The library was titered according to the Matchmaker\textsuperscript{TM} Gal4 Two-hybrid System 3 and Libraries User Manual. The colony-forming units (CFU) for the library were calculated to be about $3.1 \times 10^9$ CFU/ml. Four large scale transformations (equal to one library scale) were done at the same time following the Matchmaker\textsuperscript{TM} Gal4 Two-hybrid System 3 and Libraries User’s Manual. To calculate the efficiency of yeast two-hybrid (i.e., the number of colons obtained per $\mu$g of library plasmid DNA) and to check the expressions of “bait” and “prey” plasmids, small amounts of the solution, diluted 10X, 100X, 1000X, and 10,000X were plated in SD/-Trp-Leu 2DO (drop out) plates. The yeast two-hybrid efficiency obtained was $3 \times 10^6$ CFU per $\mu$g of library plasmid DNA. In addition, if the yeast grew on the SD/-Trp-Leu-His-Ade + 3-AT plates, the “bait” and “prey” plasmids were considered to be interacting with each other.

\textit{Small scale transformation}

The yeast two-hybrid co-transformation on a small scale for all of the direct screenings was performed following the small-scale transformation protocol in the Matchmaker\textsuperscript{TM} Gal4 Two-
hybrid System 3 & Libraries User’s Manual. The direct screens were to test whether

NlrpNLRP12 PYD + NBD and HckHCK specifically interact; identification of HckHCK F503, Q507, L510, and D511 being critical for binding with NlrpNLRP12 PYD + NBD; and the characterization of the HckHCK C-terminal 30 amino acid fragment binding to NlrpNLRP12 PYD + NBD.

Cell transfection

293T cells, THP-1 cells, U937 cells, and RAW 264.7 cells were seeded at a density of 5 x 10^5 per well in a 6-well plate. Transfection followed the Lipofectamine® 3000 reagent (Thermo Fisher Scientific) protocol with the following modifications. Briefly, 3.75 µl of Lipofectamine® 3000 was added into 125 µl of Opti-MEM® medium (Thermo Fisher Scientific). 2.5 µg of plasmid DNA (prepared by isolation through cesium chloride (CsCl) density gradient purification (612) and 5 µl of P3000TM reagent were added into another aliquot of 125 µl of Opti-MEM® medium. Then these two tubes were mixed together, and the mixed tube was incubated at room temperature for 15 min. 250 µl of this mixture was added to the cells. The cells were incubated at 37 °C with 5% CO_2 for two days until analysis.

NlrpNLRP12 stable cell lines

THP-1 cells, U937 cells, and RAW 264.7 cells were transfected with pEFRES-P-NlrpNLRP12-3FLAG plasmid, and 2.5 µg/ml puromycin (Invitrogen™ by Thermo Fisher Scientific) was added. After two weeks, the cells were considered to be stably expressing NlrpNLRP12 after screening for expression. The stable cells were “pooled” clones, and they are maintained by adding 2.5 µg/ml puromycin.
Cell lysis and immunoprecipitation

After trypsinization, cells were lysed in 500 µl of lysis buffer (1% NP-40, 4 mM Tris-HCl, pH 8.0, 150 mM NaCl). Cells were then treated with freeze-thaw cycles, i.e., frozen in the -80°C freezer for 5 min and thawed at 37°C in a water bath for 3 min, for a total of five times. Cells were then centrifuged at 12,000 x g for 10 min at 4°C. After centrifugation, the supernatant was saved at -20°C until further use.

For immunoprecipitations, cell lysates were pre-cleared by adding 40 µl of protein A/G agarose beads (Thermo Fisher Scientific) from a 50% slurry, and the sample was rotated for 1 hr at 4°C. The sample was spun at 1000 x g for 1 min. Then, 30 µl of pre-cleared cell lysate were removed and run on immunoblotting as a control. The remaining cell lysate was incubated with 1 µg anti-Heck mouse monoclonal primary antibody overnight. On the second day, 30 µl of protein A/G agarose, from a 50% slurry and previously washed with lysis buffer three times, were added to the tube, and the tube was incubated for 3 hrs at 4°C. The beads were then washed with lysis buffer, but with the NaCl concentration increased to 500 mM. The tube was briefly centrifuged at 1000 x g for 1 min., and the supernatant was removed. This step was performed for a total of 5 times. The proteins were eluted from the beads by addition of SDS-PAGE sample buffer.

Immunoblotting

Protein concentrations were determined by the Pierce™ BCA protein assay (Thermo Fisher Scientific). About 20 µg of cell lysate samples and immunoprecipitated samples were loaded onto the 8% acrylamide gel. After overnight transfer of the proteins from an SDS-gel to a 0.45
μm pore size polyvinylidene difluoride (PVDF) membrane, the membrane was blocked in for 1 hr at room temperature 5% non-fat dry milk dissolved in (Tris-buffered saline with Tween 20, pH=8.0) (TBST) (Sigma-Aldrich). Mouse monoclonal anti-FLAG antibody (diluted 1:1000) and mouse monoclonal anti-Hck antibody (diluted 1:1000) were added and the membrane incubated overnight. For detection of bands in the cell lysate, rabbit anti-mouse IgG HRP-linked secondary antibody (diluted 1:2000) (for anti-FLAG antibody) and mouse IgGκ binding protein-HRP secondary antibody (for anti-Hck antibody) (diluted 1:1000) were added, and the membrane was incubated for 1 hr at room temperature. For immunoprecipitations, Clean-Blot™ IP detection reagent (HRP) secondary antibody (1:300) (Thermo Fisher Scientific) was used. The detection signal was visualized by using enhanced chemiluminescence (ECL) reagent (Thomas Scientific, Swedesboro, NJ). Immoblots were analyzed by chemiluminescence on a Bio-Rad ChemiDoc™ Touch Gel imaging system (Bio-Rad, Hercules, CA).

Immunofluorescence

The A protocol for immunofluorescence was followed (https://www.cellsignal.com/contents/resources-protocols/immunofluorescence-general-protocol/if), with the following modifications. Coverslips were autoclaved and coated with 0.1 mg/ml of poly-D-lysine hydrobromide (mol wt 70,000-150,000) (Sigma-Aldrich). Cells were plated onto the coverslips. The cells were fixed in 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) in phosphate buffered saline (PBS) for 15 mins. Fixation was stopped by adding 50 mM NH₄Cl in PBS for 15 min at room temperature. After raising with PBS, the fixed cells were permeabilized by adding 0.5% Triton®-X-100 in PBS for 10 min. The cells were then washed with PBS three times and blocked with 0.1% of BSA in PBS at room temperature for 1
NLRP12 binds to HCK

hr. The rabbit monoclonal anti-FLAG antibody (diluted 1:100) and mouse monoclonal anti-

Hck antibody (diluted 1:100) were added to each cover slip and incubated overnight. Goat

anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 488

diluted 1:100) and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to

Alexa Fluor 555 (1:100) were added. After incubation with secondary antibodies for 1 hr, the

cells were washed five times with PBS. During the first step, DAPI (Invitrogen™ by Thermo

Fisher Scientific) was also added at 1:1000 in PBS for 2 min. Finally, the coverslips were

mounted in ProLong™ Gold Antifade mounting medium (Life Technologies Corporation by

Thermo Fisher Scientific, Eugene, OR). The stained slides were viewed on a ZEISS LSM 880

(Pleasanton, CA) with Airyscan. The images were viewed and processed using Fiji software

IPA diagram

Yeast two-hybrid data were analyzed by IPA software (QIAGEN Inc.,


Statistical analysis

The Spearman coefficient and Pearson coefficient used for co-expression was generated by

cBioPortal website (37,38,634). Clinical samples from the Oncomine website

(https://www.oncomine.org) were downloaded and checked for Nlrp12 and Hck mRNA

expression levels and Hck levels. The Fisher exact test and the odds ratio test used for co-
ocurrence were generated by the cBioPortal website. Analysis of variance (ANOVA) was

performed to check whether a significant difference of the treatment exists among all the groups.
A p-value < 0.05 was considered statistically significant. If $P < 0.05$, then the Tukey test was used to determine whether a pair-wise difference exists. Quantification of the western blot bands was performed by FIJI software (623) and made into graphs by Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Acknowledgement

Thank you to Dr. Houda Alachkar for providing valuable suggestions to this paper. We also thank Ms. Meng Li and Dr. Yibu Chen at the University of Southern California’s Norris Medical Library for their suggestions for bioinformatic analysis. Appreciation is given to Mr. Noam Morningstar-Kywi and Dr. Ian Haworth for their helps on structural analysis.

Abbreviations

3-AT: 3-Amino-1,2,4-triazole; AML: acute myeloid leukemia; ANOVA: analysis of variance; ASC: apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; ATCC: American Type Culture Collection; C1: self-named truncations of C-terminal 40 (or 41) amino acids fragment of Src-SRC family non-receptor tyrosine kinases; C2-C7 and N1-N5: self-named truncations Hck-HCK C1 fragment; CD40: cluster of differentiation 40; CDK6: cyclin D-dependent kinase; CFU: colony-forming units; COSMIC: catalogue of somatic mutations in cancer; CsCl: cesium chloride; DAMP: damage-associated molecular patterns; DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride; DC: dendritic cells; DO: drop out (2DO: SD/-Trp-Leu; 4DO: SD/-Trp-Leu-His-Ade); ECL: enhanced chemiluminescence; FAf1: Fas associated factor 1; FLT3-ITD: FLT3-internal tandem duplications; FLT3: feline McDonough sarcoma (fms)-like tyrosine kinase 3; GSK3b2:...
NLRP1p12 binds to HCK

glycogen synthase kinase 3 beta; HCK C1 fragment; Hck, HCK C-terminal 40 amino acids; HSP90, heat shock protein 90; IL, interleukin; IPA, ingenuity pathway analysis; IRAK1, IL-1 receptor-associated kinase 1; KO, knock out; KRAS, Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LRR, leucine rich repeat domain; M1, the amino acids in the R1 region were mutated to the amino acids identical to the R1 region in Lyn; M2, the amino acids in the R2 region were all mutated to Alanine; M3, the amino acids in the R3 region were all mutated to Alanine; M4, the amino acids in the R4 region were all mutated to Alanine; NBD, nucleotide binding domain; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NIK, NF-κB-inducing kinase; NLR, nucleotide binding domain and leucine-rich repeat; NLRP, NLR pyrin domain-containing protein; PAMP, pathogen-associated molecular patterns; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PDB, protein data bank; PMA, phorbol 12-myristate 13-acetate; PRR, pattern recognition receptors; PVDF, polyvinylidene difluoride; PYD, pyrin domain; R1, R2, R3, and R4, self-named four regions in the Hck, HCK C terminal 40 amino acids fragment; SD, synthetic dropout; SD/-Trp-Leu, SD base plus amino acids supplements minus tryptophan and leucine; SD/-Trp-Leu-His-Ade, SD base plus amino acids supplements minus tryptophan, leucine, and histidine, and adenine; STRING: search tool for recurring instances of neighboring genes; TBST, tris-buffered saline with Tween 20, pH=8.0; TCA, trichloroacetic acid; TCGA, the cancer genome atlas; TLR, toll-like receptor; TRAF3, tumor necrosis factor receptor associated factor 3; TRAF3IP3, TRAF3 interacting protein 3; UBA, ubiquitin-associated domains; WT, wild type; YPDA, yeast extract, peptone, dextrose, and adenine; hemisulfate.
Conflict of Competing Interest

The authors have declared that no competing conflict exists of interest regarding publication of this article.

Author Contributions Statement

YZ design the experiment and wrote and revised the manuscript. CO helped to design the experiments and to revise the manuscript.

References


31
NLRP12 binds to HCKek


phagocyte duties - from signaling and actin reorganization to migration and phagocytosis.


Table 1: yeast two hybrid results (hit frequency >= 3)

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<th>Name</th>
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<td>Deleted in azoospermia (DAZ) associated protein 2 (DAZAP2)</td>
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</tr>
<tr>
<td>Filamin A, alpha (FLNA)</td>
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</tr>
<tr>
<td>Hematopoietic cell kinase (HCK) proto-oncogene, Src-SRC family tyrosine kinase (HCK)</td>
<td>9</td>
</tr>
<tr>
<td>Chaperone DnaJ (a.k.a., heat shock protein 40 kD(Hsp40)) homolog, subfamily B, member 1 (DNAJB1)</td>
<td>9</td>
</tr>
<tr>
<td><strong>HckHCK</strong></td>
<td><strong>NlrrpNLRP</strong> family</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------</td>
</tr>
<tr>
<td><strong>HckHCK</strong></td>
<td><strong>NlrrpNLRP1</strong></td>
</tr>
<tr>
<td></td>
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<tr>
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<tr>
<td></td>
<td><strong>NlrrpNLRP6</strong></td>
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Table 2: Co-occurrence of mRNA expression level of **HckHCK** and all of the **NlrrpNLRP** family proteins (from the provisional data, assessed on July 11 2019)
Table 3: Primers used in cloning (The underline shows the restriction enzymes)

<table>
<thead>
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<th>Primers</th>
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<td>NlrpNLRP12 5’ primer</td>
<td>GATCTACATATGCTACGAACCGCAGGCAG (cloned into PGBK7)</td>
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<td>NlrpNLRP12 3’ primer</td>
<td>GATGTAATCGACTCACCCTCGTCCAGGATATAGTACATAGCT (cloned into PGBK7)</td>
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<tr>
<td>NlrpNLRP3 5’ primer</td>
<td>GATCTACATATGAAGATGGCAAGCACCACCGCTGCA (cloned into pGBK7)</td>
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<td>NlrpNLRP3 3’ primer</td>
<td>GATGTAATCGACTCACAGCAGGTAGTACATGGCGGCAAGA (cloned into PGBK7)</td>
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<tr>
<td>NlrpNLRP8 5’ primer</td>
<td>GATCTAGAATTCATGAGTGACGTGAATCCACCCCTCTG</td>
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</tbody>
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**Figure legends:**

**Figure 1:**  (A) Schematic drawing of the domain structure of full-length \textit{NlrepNLRP12} (top) and the domains used as the construct for the yeast two-hybrid screen (bottom). PYD: Pyrin domain; NBD: Nucleotide binding domain; and LRR: Leucine rich repeat domain. (B) The domain structures of the two alternatively spliced isoforms of \textit{HckHCK}, \textit{HckHCK} p59 and \textit{HckHCK} p61.  (C) Identification of the clones of \textit{HckHCK} interacting with \textit{NlrepNLRP12} (“hits”) obtained from the
yeast two-hybrid screen. Among the nine of the clones positive for HCKe, one clone encoded for the smallest interacting fragment of HCKe, the C-terminal 40 amino acids of HCKe. The numbers in parentheses indicate the total number of hits obtained in the screen.

**Figure 2**: Specificity of binding of Nlrp12 with HCKe. (A) Schematic drawing of HCKe (p61 isoform, top) and, for comparison, the region of HCKe (“HCKe C1 fragment,” lower) used for testing the specificity of binding of HCKe to other members of the Nlrp family of proteins. The numbers represent the amino acid residues of the protein or protein fragment (B) Phylogenetic tree showing the relationships among the PYD + NBD region of all of the members of the human Nlrp family of proteins. The phylogenetic tree was produced through Clustal Omega ([https://www.ebi.ac.uk/Tools/msa/clustalo/](https://www.ebi.ac.uk/Tools/msa/clustalo/)). For each Nlrp family member, the PYD + NBD of the longest isoform was selected for generating the phylogenetic tree. Nlrp3 is phylogenetically the closest Nlrp to Nlrp12, while Nlrp8 is phylogenetically among the most distant from Nlrp12. Nlrp3 and Nlrp8 are highlighted in red color. (C) When Nlrp12’s PYD + NBD and HCKe C1 fragment were co-transformed into yeast, they grew on the highest stringency plates (4DO + 3AT), consistent with an interaction between the two constructs. On the other hand, neither Nlrp3’s PYD + NBD co-transformed with the C1 fragment nor Nlrp8’s PYD + NBD co-transformed the C1 fragment grew on the highest stringency plates. These results are consistent with a specific interaction between Nlrp12 and the HCKe C1 fragment, but not with the other Nlrp families. (D) Yeast clones grew on the high stringency plates 4DO + 3AT when HCKe’s C1 fragment and Nlrp12’s PYD + NBD were co-transformed, but they did not grow on the high stringency 4DO + 3AT plates when the C1 fragments of other members of
the Sc-SRCrc family of non-receptor tyrosine kinases were co-transformed with \textit{NlrpNLRP12}'s \textit{PYD + NBD}. These results are again consistent with a specific interaction between \textit{NlrpNLRP12} and HCKek.

\textbf{Figure 3:} (A) Structure of the HCKek C1 fragment generated by Chimera from the University of California, San Francisco (version 1.13.1), extracted from its structure within the entire HCKek protein. HCKek C1 fragment was thus divided into four helical domains: R1, R2, R3, and R4. The side chains of amino acids F503, I506, Q507, L510, and D511 are shown. However, it is not clear whether the C1 fragment retains the structural features shown here when it expressed on its own. (B) Primary amino acid sequence alignments showing of the C1 fragments of all members of the Sc-SRCrc non-receptor tyrosine kinase family (40 amino acids for HCKek, BKlk, LCKek, and LYNyn; 42 amino acids for FGRgr, Sc-SRCrc, YESes, and FYNyn). The black lines indicate the R1, R2, R3, or R4 regions. The arrows denote the four critical amino acids for binding of HCKek C1 fragment to \textit{NlrpNLRP12}'s PYD + NBD. (C) Phylogenetic tree showing the relationship among all of the C1 fragments of the members of the Sc-SRCrc family of non-receptor tyrosine kinases. The phylogenetic tree was produced through Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). HCKek is was highlighted in red color. (D) Four sets of distinct mutations were made within the HCKek C1 fragment. M1: the amino acids in the R1 region were mutated to the amino acids identical to the R1 region in LYNyn (highlighted in red color); M2: the amino acids in the R2 region were all mutated to alanine (Ala); M3: the amino acids in the R3 region were all mutated to Ala; and M4: the amino acids in the R4 region were all mutated to Ala. (E) Yeast did not grow on high stringency 4DO + 3AT plates when \textit{HCKek C1 fragment} with the M3 mutations was co-transformed with \textit{NlrpNLRP12}'s \textit{PYD + NBD}.
NLRP12 binds to HCK

NBD. But yeast can grow on the high stringency 4DO + 3AT plates when the HCK C1 fragment contained either the M1, M2, or M4 mutations, suggesting that the R3 region within the C1 fragment was necessary for binding to NlrpNLRP12 NBD + PYD. (E) Yeast did not grow on high stringency 4DO plates when NlrpNLRP12’s PYD + NBD was co-transformed with HCK C1 fragment with single Ala substitutions at either F503, I506, Q507, L510, or D511. But yeast grew on high stringency 4DO plates when NlrpNLRP12’s PYD + NBD was co-transformed with HCK’s C1 fragment in which the following amino acids in the R3 region were individually mutated to Ala: F504, Y505, S508, or V509. Yeast did not grow on either 2DO nor 4DO plates when NlrpNLRP12’s PYD + NBD was co-transformed with Hck C1 fragment in which either E504 or I506 was individually mutated to Ala, making the results with these particular mutants inconclusive.

Figure 4: (A) Top: Schematic drawing of HCK truncations comprising the C1 to C7 fragments. Bottom: Yeast grew on the high stringency 4DO plates and 4DO +3AT plates when HCK’s C-terminal 40 amino acid fragment (C1), HCK’s C-terminal 35 amino acid fragment (C2), and HCK’s C-terminal 30 amino acid fragment (C3), individually, were co-transformed with NlrpNLRP12’s PYD + NBD. But yeast did not grow on the high stringency 4DO plates nor 4DO+ 3AT plates when C4 to C7 fragments were co-transformed to with NlrpNLRP12’s PYD + NBD. The lack of yeast growth upon co-transformation of the C7 fragment and NlrpNLRP12 are not shown. (B) Top: Schematic drawing of Hck truncations comprising the N1 to N5 fragments. Bottom: Yeast grew on the high stringency 4DO plates and 4DO +3AT plates when HCK’s C-terminal 40 amino acid fragment (C1) was co-transformed with NlrpNLRP12’s PYD + NBD (control). But yeast did not grow on the high
stringency 4DO plates nor 4DO+ 3AT plates when \textit{N1 and N5 fragments} were co-transformed to \textit{NlrpNLRP12}'s PYD + NBD, suggesting that the very C-terminal 5 amino acids are also necessary for binding of \textit{NlrpNLRP12}. The colored boxes represent different fragments that were deleted each time.

\textbf{Figure 5}: \textit{NlrpNLRP12} co-immunoprecipitates with \textit{HCKek} and co-localizes with \textit{HCKek} by immunofluorescence. (A) \textit{NlrpNLRP12} co-immunoprecipitated with \textit{HCKek} when both proteins were transiently exogenously co-expressed in 293T cells, with \textit{NlrpNLRP12} having to be expressed as epitope-tagged forms. Cells were lysed using a non-denaturing cell lysis buffer (see Material and Methods). Co-immunoprecipitations were done using a mouse monoclonal anti-\textit{HCKek} antibody followed by protein A/G agarose. The immunoprecipitated proteins were analyzed by immunoblotting. The same method was used for experiments shown in (B), (C), and (D). (B) \textit{NlrpNLRP12} co-immunoprecipitated with endogenous \textit{HCKek} in macrophage-like RAW 264.7 cells, when \textit{NlrpNLRP12} is exogenously and stably expressed. Arrows show the two isoforms of \textit{HCKek} p59 and p61 that were immunoprecipitated by the anti-\textit{HCKek} antibody, although it is not clear whether \textit{NlrpNLRP12} co-immunoprecipitated with one form or the other, or both. (C) Exogenously and stably expressed \textit{NlrpNLRP12} co-immunoprecipitated with endogenously expressed \textit{HCKek} in monocyte-like THP-1 cells. In these cells, phorbol 12-myristate 13-acetate (PMA) (1µM) was added to enhance the expression of \textit{NlrpNLRP12} under the PMA-sensitive cytomegalovirus promoter of the \textit{NlrpNLRP12} plasmid. (D) Left panel shows that \textit{NlrpNLRP12} was exogenously and stably expressed in lymphoblast-like U937 cells. Right panel shows that \textit{NlrpNLRP12} co-immunoprecipitated with endogenously expressed \textit{HCKek} in U937 cells after PMA was added to 1µM, as was done for THP-1 cells, to enhance
expression of NlrpNLRP12. (E) Immunofluorescent images showing the colocalization of a cohort of NlrpNLRP12 with either HCKek p59 or p61. HCKek was immunolabeled with a mouse monoclonal anti-HCKek antibody, followed by a secondary goat anti-mouse IgG (H+L) cross-adsorbed, conjugated to Alexa Fluor 488. For NlrpNLRP12, the primary antibody was a rabbit monoclonal anti-FLAG antibody, and the secondary antibody was a goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555. Nuclei were counterstained by DAPI.

**Figure 6:** The co-expression of HCKek protein significantly decreased the steady-state protein expression levels of NlrpNLRP12 (A) Epitope-tagged NlrpNLRP12-V5 and HCKek p61 were co-transfected into 293T cells. Cell lysates were immunoblotted with rabbit monoclonal anti-V5 antibody for NlrpNLRP12 and mouse monoclonal anti-HCKek antibody. Comparing lane 2 (basal level of NlrpNLRP12 expression) with lanes 5, 6, and 7, co-expression of HCKek protein leads to a significant decrease of protein expression levels of NlrpNLRP12 over three times they are co-expressed. Increasing protein expression of HCKek, relative to the plasmid DNA added (ramp: lanes 5, 6, and 7; 2.5-20 μg of DNA) was not observed possibly because the amount of plasmid for HCKek transfection were already too high, resulting in the maximum expression of HCKek at all plasmid DNA amounts. The figure is a representative of one of the results from three experiments. (B) Quantification of the NlrpNLRP12 protein bands (mean + standard error) to show that NlrpNLRP12 protein expression levels decreased in the presence of co-expressed HCKek, relative to equal amounts of cell lysate being assayed. The NlrpNLRP12 protein expression levels are indicated as arbitrary units. (C) Quantification of the HCKek protein bands (mean + standard error) confirming that HCKek protein expression levels
did not change although the amount of plasmid DNA for \textit{HCK Hck}-increased. Figure (B) and (C) were generated by Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Twenty µg of cell lysate proteins were loaded for each lane. ANOVA and Tukey tests were used to compare the \textit{NlrpNLRP12} and \textit{HCKek} protein bands.

\textbf{Figure 7:} \textit{NlrpNLRP12} is co-expressed with \textit{Hck} in acute myeloid leukemia (AML) patient samples. Both \textit{NlrpNLRP12} expression and \textit{Hck} expression data are shown as log2 values. The data are RNA seq data obtained from cBioportal (provisional) database assessed on July 11, 2019. The graph was taken from the cBioportal website. Spearman coefficient, indicating the association of \textit{NlrpNLRP12} and \textit{Hck} mRNA expression level (if \textit{NlrpNLRP12} and \textit{Hck} mRNA expression levels are non-parametric) = 0.80. Pearson coefficient, indicating the degree of the linear relationship between the \textit{NlrpNLRP12} mRNA expression level and \textit{Hck} mRNA expression level (if \textit{NlrpNLRP12} and \textit{Hck} mRNA expression levels are parametric) = 0.73. The p-values for Spearman coefficient and Pearson coefficient are both < 0.001.

\textbf{Figure 8:} (A) Ingenuity Pathway Analysis (IPA) displaying the 13 distinctive hits from the total results of the yeast two-hybrid screen for proteins that interacted with \textit{NlrpNLRP12}. The figure only shows the \textit{NlrpNLRP12}-interacting proteins with hits that have a frequency larger than 2. The solid lines indicate interacting proteins found through our yeast two-hybrid screen. The dashed lines are the \textit{NlrpNLRP12}-interacting proteins previously reported in published data. The purple-colored molecules are those associated with AML. (B) To validate an interaction shown in the IPA diagram, tumor necrosis factor receptor-associated factor 3 interacting protein 3 (TRAF3IP3)-3FLAG and \textit{NlrpNLRP12-V5} were co-immunoprecipitated from co-transfected
NLRP12 binds to HCKck

293T cells. A non-denaturing cell lysate buffer (see Material and Methods) was used to lyse the cell, and co-immunoprecipitations were performed with a mouse monoclonal anti-TRAF3IP3 antibody followed by protein A/G agarose. The immunoprecipitates were analyzed for NLRP12 by immunoblotting with rabbit monoclonal anti-V5 antibody. The results from the co-immunoprecipitation are consistent with a confirmation of an interaction between TRAF3IP3 and NLRP12.
NLRP12 binds to HCK.
Figure 1
NLRP12 binds to HCKeck

(A) P61 Hck

(B) Nlrp11
   - Nlrp5
   - Nlrp7
   - Nlrp2
   - Nlrp1
   - Nlrp6
   - Nlrp10
   - Nlrp12
   - Nlrp3
   - Nlrp13
   - Nlrp14
   - Nlrp9
   - Nlrp4

(C) Vector
   - C1 fragment

   Nlrp12’s PYD + NBD
   Nlrp3’s PYD + NBD
   Nlrp8’s PYD + NBD

   2DO  4DO  4DO + 3AT

(D) Vector
   - Hck-CI
   - Lyn-CI
   - Lck-CI

   Nlrp12’s PYD + NBD

   2DO

   Vector
   - Fgr-CI
   - Src-CI
   - Yes-CI
   - Fyn-CI

   4DO + 3AT
**Figure 2**

(A) P61 HCK

(B) NLRP11
NLRP5
NLRP7
NLRP2
NLRP1
NLRP6
NLRP10
NLRP12
NLRP3
NLRP13
NLRP8
NLRP14
NLRP9
NLRP4

(C) Vector
Cl fragment

(NLRP12’s PYD + NBD)
(NLRP3’s PYD + NBD)
(NLRP8’s PYD + NBD)

(D) Vector
HCK-Cl
LGN-Cl
LSK-Cl

(NLRP12’s PYD + NBD)

2DO
4DO
4DO + 3AT

(NLRP12’s PYD + NBD)

Vector
NLRP12p12 binds to HCK

Figure 3
**Figure 3**

**A**

**B**

**C**

**D**

**E**

**F**

NLRP12 binds to HCKek

Vector

NLRP12’s PYD + NBD

2DO

4DO

HCK C-terminal 40 a.a.

Vector

NLRP12’s PYD+NBD

2DO

4DO

Vector

NLRP12’s PYD+NBD

2DO

4DO
NLRP1p12 binds to HCKe

(A) C1 (a.a. 486-525) H,N α-helix

N1 (a.a. 496-520) H,N
N2 (a.a. 496-515) H,N
N3 (a.a. 496-510) H,N
N4 (a.a. 496-505) H,N
N5 (a.a. 496-500) H,N

Vector Nlrp12’s Pyrin + NBD PYD alone NBD alone

2DO 4DO 4DO + 3AT

(B) C1 (a.a. 486-525) H,N α-helix

C2 (a.a. 491-525) H,N
C3 (a.a. 496-525) H,N
C4 (a.a. 501-525) H,N
C5 (a.a. 506-525) H,N
C6 (a.a. 511-525) H,N
C7 (a.a. 516-525) H,N

Vector Nlrp12’s PYD + NBD PYD alone NBD alone

2DO 4DO 4DO + 3AT
Figure 4

NLRP12 binds to HCK.
**A** 293T overexpression

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**B** Vector/Raw264.7 stable cell line

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**C** Vector/THP-1 stable cell line

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**D** Vector/U937 stable cell line

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**E** U937 stable cell line

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**F**

- **Hck p59**
- **Hck p61**
- **DAPI**
- **Anti-Hck**
- **Anti-FLAG (Nlrp12)**
- **Merge**
Figure 5

NLRP12 binds to HCK.
NLRP12 binds to HCKc

(A) NLRP12-V5 - + - + + +
HCK p61 - + + -
Nlrp12-V5 - - - -
Hck - - - -
Lane 1 2 3 4 5 6 7
αV5
αHck

(B) Nlrp12 expression levels

(C) Hck protein expression levels
Figure 6

(A) NLRP12-V5 and HCK p61

(B) NLRP12 protein expression levels

(C) HCK protein expression levels

NLRP12 binds to HCK

Lane 1 2 3 4 5 6 7
NLRP12 binds to HCK

Spearman coefficient = 0.80
(p value < 0.001)

Pearson coefficient = 0.73
(p value < 0.001)
NLRP12 binds to HCK.

Figure 7

Spearman coefficient = 0.80
(p value < 0.001)

Pearson coefficient = 0.73
(p value < 0.001)
NLRP12 binds to HCK

(A) 

Legend:
- Enzyme
- Kinase
- Transmembrane receptor
- Transcriptional regulator
- Other

Molecules associated with AML

Protein-protein interactions from IPA

Protein-protein interactions from Y2H

(B)

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IP: TRAF3IP3

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WCL

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NLRP12 binds to HCK

Figure 8
Nucleotide binding domain and leucine-rich repeat pyrin domain-containing protein 12: characterization of its binding to hematopoietic cell kinase

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Running title: NLRP12 binds to HCK

Key words: yeast two-hybrid, innate immunity, protein-protein interaction, NLRP12, HCK, acute myeloid leukemia

Abstract
Protein-protein interactions are key to defining the function of nucleotide binding domain (NBD) and leucine-rich repeat (LRR), pyrin domain (PYD)-containing family of proteins 12 (NLRP12). cDNA encoding the human PYD + NBD of NLRP12 was used as bait in a yeast two-hybrid
screen with a human leukocyte cDNA library as prey. Hematopoiesis cell kinase (HCK), a member of the c-SRC family of non-receptor tyrosine kinases, was among the top hits. The C-terminal 40 amino acids of HCK specifically bound to NLRP12’s PYD + NBD, but not to that of NLRP3 and NLRP8. Amino acids F503, I506, Q507, L510, and D511 of HCK are critical for the binding of HCK’s C-terminal 40 amino acids to NLRP12’s PYD + NBD. Additionally, the C-terminal 30 amino acids of HCK are sufficient to bind to NLRP12’s PYD + NBD, but not to its PYD alone nor to its NBD alone. In cell lines that express HCK endogenously, it was co-immunoprecipitated with stably expressed exogenous NLRP12. Also, NLRP12 co-immunoprecipitated and co-localized with HCK when both were overexpressed in 293T cells. In addition, in this overexpression system, steady-state NLRP12 protein expression levels significantly decreased when HCK was co-expressed. Bioinformatic analysis showed that HCK mRNA co-occurred with NLRP12 mRNA, but not with other NLRP mRNAs, in blood and marrow samples from acute myeloid leukemia (AML) patients. The mRNA of NLRP12 is also co-expressed with HCK in AML patient samples, and the levels of mRNA expression of each are correlated. Together these data suggest that NLRP12, through its binding to HCK, may have an effect on the progression of AML.

Introduction

Pattern recognition receptors (PRR) are the first major line of cellular defense against damage-associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP) (1). Among PRR, one class of proteins is the nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) receptor family (NLR). NBD and LRR PYD protein 12 (NLRP12) is one protein that belongs to the PYD subfamily of the NLR class and is a multi-functional protein (2).
NLRP12 binds to HCK

NLRP12’s functions likely all depend on defined protein-protein interactions. For example, in anti-inflammatory functions of NLRP12, NLRP12 interacts with tumor necrosis factor receptor associated factor 3 (TRAF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-inducing kinase (NIK) in the non-canonical NF-κB pathway, when toll-like receptors (TLR) are stimulated with Pam3Cys4 triacylated lipopeptides followed by addition of cluster of differentiation 40 (CD40) (3). Kanneganti’s group also showed that NLRP12 expression downregulated the canonical NF-κB pathway during Salmonella infection (4,5). NLRP12’s inhibition of the non-canonical NF-κB pathway, thereby effecting an anti-inflammatory function, has been reported in many publications (7-11). For example, in colitis-driven colon cancer, Nlrp12 knock-out (KO) mice experience a higher frequency of death, weigh less, have a shorter colon length, and have more severe disease progression than wild type (WT) mice, all of which may occur through NLRP12’s inhibition of the non-canonical NF-κB pathway (3,4). In addition, Nlrp12 KO mice have been reported to exhibit higher innate immunity when it is infected by the virus because NLRP12 inhibits the non-canonical NF-κB pathway; thus, in a KO mice, a more significant increase of NF-κB activity is observed compared to WT mice when it is infected by the virus (6). And in the case of NLRP12 protecting the diversity of intestinal microbiota (7), thereby preventing obesity (8), it has also been reported that Nlrp12 KO mice display significantly decreased intestinal microbial diversity due to increased innate immunity, resulting from the lack of inhibition of the non-canonical NF-κB pathway.

In addition, NLRP12 also interacts with interleukin (IL)-1 receptor-associated kinase 1 (IRAK1) in the canonical NF-κB pathway when TLRs are stimulated, also to inhibit the inflammatory response (9). In contrast, for human studies of NLRP12-associated autoinflammatory disorders,
NLRP12 binds to HCK

no protein-protein interactions have been reported (10,11). Functionally, however, Nlrp12 KO mice had a higher immunological response in the autoimmune model of contact hypersensitivity (12); thus, these mice are more prone to develop contact hypersensitivity. This results most likely from Nlrp12 KO mice having defective migration of their dendritic cells (DC). In addition, Luken et al. demonstrated that NLRP12 impacts the experimental autoimmune encephalomyelitis (EAE) model via regulating T-cell responses (13). But, in these mouse studies, no protein-protein interactions had been reported either (12). And NLRP12 is protective against Yersinia pestis infection through NLRP12’s activation of the inflammasome which results in secretion of IL-18 and IL-1β. But, no protein-protein interactions of NLRP12 within inflammasomes had been characterized (14). Also, NLRP12 also regulates the c-Jun N-terminal kinase (JNK) pathway in hepatocytes (15); but, again, no protein-protein interactions within the NLRP12-JNK pathway had been demonstrated. Thus, there is a further need to find and to characterize previously unreported NLRP12-binding proteins. Knowing NLRP12’s protein binding partners may help to gain mechanistic insight into pathways previously characterized as being regulated by NLRP12. Moreover, identifying and validating potentially novel binding partners of NLRP12 may suggest novel functions for NLRP12.

In humans, NLRP12 is mainly expressed in macrophages, DCs, and neutrophils. In mice, NLRP12 is primarily expressed in DCs and neutrophils (6,12,16). The NLRP12 protein has three domains, from N- to C-terminus: PYD, NBD, and LRR domains (2,17,18). It is generally accepted that the LRR domain, like NLRP1’s LRR domain and NLRP3’s LRR domain, is responsible for sensing ligands or other types of activators of NLRPs in modulating their role in an immunologic response (19-22). However, interestingly, it has been reported that without the
NLRP12 binds to HCK

LRR domain, NLRP3 still can be activated by triggers of the canonical NF-κB pathway (23).

The NBD domain is responsible for self-oligomerization when NLRP is activated, and the PYD domain is responsible for regulating downstream signaling pathways (and thus, it is called the “interaction domain”) (16,23). Here we used a truncated form of NLRP12 (PYD + NBD) to identify possible protein-protein interactions that may regulate signaling pathways in which NLRP12 is involved. In addition, to our knowledge, there are currently no protein-protein interaction studies that have been performed for this truncated form of NLRP12 and may thus represent a new general approach to screening for protein-protein interactions involved in signaling complexes with NLRPs.

A yeast two-hybrid screen was performed to identify potential binding partners of NLRP12’s PYD + NBD. HCK, a member of the c-SRC family of non-receptor tyrosine kinases, was one of the top positive clones (“hits”) among all of the proteins that appeared to interact with NLRP12. Further experiments were done to confirm the novel binding of NLRP12 to HCK and to characterize key structural features of the binding interaction. In addition, co-expression of HCK with NLRP12 resulted in a significant decrease of steady-state NLRP12 protein expression levels. Finally, together with a bioinformatics analysis included here, a potential interaction between NLRP12 and HCK may be involved in modulating the role of HCK in the pathogenesis of AML (24-26).

Results

NLRP12 interacts with HCK in the yeast two-hybrid assay
A human leukocyte cDNA library was used as prey in the screening of binding partners for NLRP12 using the yeast two-hybrid assay. The basic principle of the yeast two-hybrid assay is that if the cDNA library contains clones that express proteins, or parts thereof, that interact with the co-expressed bait, the NLRP12 PYD + NBD (Figure 1A) in this case, then the co-transformed yeast clones will grow. The cDNA clones from the library that supported growth would then be isolated and characterize, to identify what proteins interact with NLRP12’s PYD + NBD. The leukocyte library was used because NLRP12 is mainly expressed in leukocytes in humans (2,16,27,28), and thus putative binding partners for NLRP12 are also likely to be expressed there. The screening resulted in 119 positive clones from the leukocyte cDNA library (“hits”) expressed from 48 distinct genes (Supplementary materials, Table S1) that were subsequently identified as encoding for proteins (or portions thereof) that potentially interact with NLRP12’s PYD + NBD. The clones with the most hits are listed in Table 1. Among the 119 hits, which included repeated hits of the same genes, 9 were individual clones that encoded fragments of a member of the c-SRC family of non-receptor tyrosine kinases, HCK, which was among the highest number of hits for a single gene (Figure 1B and Table 1). Moreover, of the 9 positive HCK clones, 5 encoded distinct HCK fragments (Figure 1C). Full-length HCK p61 was also obtained as a hit in another independent yeast two-hybrid screen (data not shown). HCK has two isoforms, p59 and P61, in Homo sapiens. P61 and p59 share the same transcript, but p61 uses an uncommon start codon CTG, and p59 uses the common one, ATG (29), to give rise to two distinct transcripts. Endogenously expressed in phagocytes, HCK p59 is mainly localized to the plasma membrane, while HCK p61 is primarily localized to lysosomes, and both of them are also found in the Golgi apparatus (30). It has been suggested that overexpression of p59 promotes the formation of membrane protrusions such as pseudopodia and overexpression of p61
NLRP12 binds to HCK

induces podosome rosettes and triggers actin polymerization around lysosomes (30,31).

However, there are no differences between p61 and p59 in the region of HCK that appears to bind to NLRP12’s PYD + NBD (see below), the C-terminal part of HCK. Thus, detecting a full-length clone of only p61 as a hit, opposed to p59, appeared to be coincidental.

In directed screens using the yeast two-hybrid assay, NLRP12’s PYD + NBD domain binding to HCK C-terminal 40 amino acids appears to be selective for NLRP12, and HCK binding to NLRP12 appears to be selective for HCK

Initially, the HCK C-terminal 40 amino acids (“HCK C1 fragment”) was the shortest HCK fragment from a positive clone that interacted with the bait, NLRP12’s PYD + NBD (Figure 2A).

NLRP3 and NLRP8 were chosen based on the phylogenetic trees of the PYD + NBD domains of members of the NLRP family: phylogenetically, NLRP3’s PYD + NBD is the most closely related NLRP PYD + NBD to that of NLRP12, while NLRP8’s PYD + NBD is among the furthest related PYD + NBD to that of NLRP12 (Figure 2B). In Figure 2C, yeast clones grew on the 4 drop out (DO) (synthetic dropout (SD) plus amino acids supplements minus tryptophan, leucine, histidine, and adenine (-Trp-Leu-His-Ade)) and 4DO + 3-Amino-1,2,4-triazole (3-AT) high stringency plates when the yeast were co-transformed with NLRP12’s PYD + NBD and HCK C1 fragment; and thus, this result is indicative of an interaction between the two constructs. However, yeast clones were not observed on 4DO and 4DO + 3-AT plates when NLRP3’s PYD + NBD and HCK C1 fragment were co-transformed, nor when NLRP8’s PYD + NBD and HCK C1 fragment were co-transformed. These results suggest that the HCK C1 fragment binds to NLRP12’s PYD + NBD, but not to those from either NLRP3 or NLRP8, results consistent with a selective interaction between NLRP12’s PYD + NBD and the HCK C1 fragment.
NLRP12 binds to HCK

Similarly, yeast clones again grew on the high stringency 4DO and 4DO + 3-AT plates when the
HCK C1 fragment was co-transformed with NLRP12’s PYD + NBD. However, clones did not
grow on the 4DO and 4DO + 3-AT plates when the C1 fragment of other members of the c-SRC
family of non-receptor tyrosine kinases were co-transformed with NLRP12’s PYD + NBD
(Figure 2D). This experiment suggests that the interaction of HCK C1 fragment with NLRP12 is
selective for HCK; the C1 fragment of other members of the c-SRC family of non-receptor
tyrosine kinases did not bind to NLRP12’s PYD + NBD (Figure 2D).

In directed screens using the yeast two-hybrid assay, amino acids F503, I506, Q507, L510,
and D511 of HCK are critical for the binding between NLRP12’s PYD + NBD and HCK C-
terminal 40 amino acids

The HCK C1 fragment were divided into four helical regions according to its atomic structure
(32) (protein data bank (PDB) accession number: 1AD5): R1, R2, R3, and R4 (designated by us)
(Figure 3A). As shown in the sequence alignments in Figure 3B, the primary amino acid
sequences of R2, R3, and R4 are very well conserved among members of the c-SRC family of
non-receptor tyrosine kinases, while R1 is not. To characterize further the important structural
features of HCK’s binding to NLRP12’s PYD + NBD, all of the amino acids comprising each of
the R2, R3, and R4 domains were mutated to alanine, but only one section at a time was mutated,
and these were denoted as the HCK M2 fragment, HCK M3 fragment, and HCK M4 fragment,
respectively. The amino acids comprising the R1 region were mutated to those amino acids in
the R1 region of the c-SRC family non-receptor tyrosine kinase LYN, which has the closest
phylogenetic relationship to HCK (Figure 3C). All of the mutated constructs are illustrated in
Figure 3D. Yeast still grew on the high stringency 4DO + 3AT plates when NLRP12’s PYD + NBD was co-transformed with the HCK M1 fragment, HCK M2 fragment, and HCK M4 fragment. But, yeast could not grow when NLRP12’s PYD + NBD was co-transformed with HCK M3 fragment (Figure 3E). These experiments suggested that the R1, R2, and R4 regions are dispensable for binding, while R3 may be important for binding. Further analysis was conducted to identify individual critical amino acids of HCK’s R3 region binding to NLRP12’s PYD + NBD using alanine scanning mutagenesis. However, the intervening sequences between R1, R2, R3, and R4 were not examined for binding.

To identify the key amino acid residues involved in binding of R3 to NLRP12’s PYD + NBD, each amino acid in R3 was individually mutated to alanine in an alanine scanning mutagenesis analysis, on the background of the HCK C1 fragment. As shown by the yeast two-hybrid assay, the mutations of F503A, I506A, Q507A, L510A, and D511A abolished the growth of yeast clones on the high stringency 4DO when these yeast strains were co-transformed with NLRP12’s PYD + NBD (Figure 3F). These experiments suggest that these four amino acids are critical for binding. On the other hand, yeast clones still grew on the high stringency 4DO plates when the HCK C1 fragments with the E504A, Y505A, S508A, or V509A mutations were co-transformed with NLRP12’s PYD + NBD (Figure 3F). These experiments suggest that E504A, Y505, S508, and V509 are dispensable for binding of HCK C1 fragment to NLRP12’s PYD + NBD. Overall, however, these results suggest that the amino acids F503, I506, Q507, L510, and D511 in HCK are critical for the binding of HCK C1 fragment to NLRP12’s PYD + NBD.
On the other hand, YES also has these five amino acid residues, (Figure 3A), suggesting that, while they are important for binding, they cannot account for the specificity or selectivity of binding between NLRP12 and HCK. In addition, the amino acids R500, P501, T502, D512, T515, Q520, and Y521, are 100% conserved among all other members of the c-SRC family of non-receptor tyrosine kinases and are therefore unlikely to play a role in specificity and selectivity of binding of NLRP12 with HCK. But, it is possible that two or more amino acids in the intervening sections between the R1-R4 regions, which were not analyzed by mutagenesis, may then actually account for the specificity and selectivity of binding of HCK to NLRP12. One example is that the combination of R496, P497, and Q522 is unique to HCK compared to other members of the c-SRC family of non-receptor tyrosine kinases.

**In directed screens using the yeast two-hybrid assay, the shortest fragment of HCK that binds to NLRP12’s PYD + NBD is its C-terminal 30 amino acids**

The shortest form of HCK that bound to NLRP12’s PYD + NBD was determined by observing whether the yeast clones grew on high stringency 4DO or 4DO +3AT plates when NLRP12’s PYD + NBD was co-transformed with HCK fragments C1 to C7 and N1 to N5 (Figure 4) (data of NLRP12’s PYD + NBD co-transformed with HCK fragment C7 not shown). HCK’s C1 to C7 fragments were gradually truncated from the N-terminus of the HCK C-terminal 40 amino acid fragment (Figure 4A). HCK N1 to N5 fragments were gradually truncated from the C-terminus of the HCK C-terminal 40 amino acid fragment (Figure 4B). Yeast strains grew on high stringency 4DO or 4DO +3AT plates when either the HCK C1, C2, or C3 fragment was co-transformed with NLRP12’s PYD + NBD. Thus, of all of the constructs screened, the shortest fragment of HCK that can apparently bind to NLRP12’s PYD + NBD is the C-terminal 30 amino
NLRP12 binds to HCK

acids, as indicated by the HCK C3 fragment (Figure 4). In addition, the last C-terminal 5 amino acids of the HCK C-terminus also appear to be important for binding, as clones co-transformed with NLRP12’s PYD + NBD with N1, with a deletion of the C-terminal 5 amino acids of HCK, did not grow on the high stringency 4DO and 4DO + 3AT plates (Figure 4B). The structures of both inactive HCK (PDB accession number: 1AD5) and active c-SRC (PDB accession number: 1Y57), the prototypical member of this family of nonreceptor tyrosine kinases related to HCK, show that the last C-terminal 4 amino acids of c-SRC are surface-exposed, indicating that these four amino acids that are critical for binding are available to interact with NLRP12 (33,34).

NLRP12 co-immunoprecipitates with HCK from mammalian cells, and NLRP12 is co-localized with HCK in mammalian cells

NLRP12 and HCK could be co-immunoprecipitated when they were both heterologously co-expressed in 293T cells (Figure 5A). Also, semi-endogenous conditions were used for co-immunoprecipitations from cells in which HCK is endogenously expressed, and NLRP12 was heterologously stably expressed in epitope-tagged form, such as in macrophage-like RAW 264.7 cells (Figure 5B), monocyte-like THP-1 cells (Figure 5C), and lymphocyte -like U937 cells (Figure 5D). Semi-endogenous conditions have been used in previous reports where NLRP12 was shown to interact with TRAF3 (3), NIK (35), and IRAK1(9). In all cases, NLRP12 co-immunoprecipitated with the p59 and p61 isoforms of HCK, but from these experiments, it is not clear that whether NLRP12 interacts with p59, p61, or both isoforms of HCK. In U937 cells and THP-1 cells, since heterologous NLRP12 expression level was low, adding phorbol 12-myristate 13-acetate (PMA) (1 µM for 24 hr) increased NLRP12 expression level by stimulating the PMA-sensitive cytomegalovirus promoter of NLRP12’s expression plasmid (Figure 5C and 5D).
Taken together, these data suggest that NLRP12 and HCK (either p59, p61, or both isoforms) can interact with each other in a mammalian cell system, a result that in part validates the findings from the yeast two-hybrid assay.

After heterologous co-expression of NLRP12 with either the p61 or the p59 isoform of HCK in 293T cells, immunofluorescent staining of NLRP12 and HCK showed that NLRP12 can be co-localized with either p61 or p59 isoforms of HCK in most of the 293T cells (Figure 5E and Supplementary materials, Figure S1). The co-localization of NLRP12 and HCK is consistent with an intracellular interaction between the two proteins. In addition, the lack of selectivity of NLRP12 co-localizing with either p61 or p59 isoform of HCK is consistent with the results from the screening assay, where the putative binding region of HCK to NLRP12 is identical in both the p61 and p59 isoforms.

The steady-state level of NLRP12 protein may decrease when HCK protein is co-expressed

Figure 6 shows that in 293T cells, the co-expression of increasing amount of HCK may led to a decrease in steady-state protein expression levels of NLRP12. Although, alternatively, the apparent decrease in expression level of NLRP12 might be due actually to a decrease in the epitope-tag itself or a decrease in the expression of the epitope-tagged protein only. Interestingly, HCK protein levels did not show a linearly increasing level of expression upon transfection with increasing amounts of plasmid DNA, possibly due to HCK’s maximum expression levels in these cells being achieved with the lowest amounts of plasmid DNA, which may be resolved if the transfections are performed with lower amounts of plasmid DNA. This lack of linearity in protein expression could also be due to saturation of the signal for HCK on the blot at relatively
NLRP12 binds to HCK

low levels of HCK expression or a combination of HCK’s maximum expression levels has been reached as well as saturation of the blot, as mentioned above. Alternatively, reduction of NLRP12 expression could be due to plasmid competition; that is, since the expression of both genes from either plasmid are driven from the same promoter, an increase in the amount of one plasmid may competitively decrease expression of the gene from the other plasmid due to their competition for transcription factors.

In blood and marrow samples from patients with AML, NLRP12 and HCK co-occur and are co-expressed

The odds ratio test (a statistical test that defines the likelihood of association of two events) determines whether two proteins either co-occur or are mutually exclusive in their expression. The Fisher exact test is a statistical test that uses P-values to determine if two events, such as co-occurrence or mutual exclusivity, are quantitatively significantly associated. Applying these tests to The Cancer Genome Atlas (TCGA) provisional data sets for AML patients (36-38), NLRP12 and NLRP1 are the only two NLRP family members that show a statistically significant co-occurrence with HCK (Table 2). However, NLRP1’s co-occurrence has a P value of 0.048, while NLRP12’s co-occurrence has a P value of <0.001. Therefore, NLRP12 is most likely the only NLRP family mRNAs that shows co-occurrence with HCK in samples from patients with AML.

Moreover, in this analysis, NLRP12 is significantly co-expressed with HCK (Pearson coefficient = 0.80 and Spearman coefficient = 0.73, Pearson and Spearman coefficients show the possibility of a linear relationship of two factors, Figure 7). This analysis is also consistent with NLRP12
NLRP12 binds to HCK

and HCK co-occurring in the same patients with AML. However, NLRP12 was not the only
NLRP mRNA that shows significant co-expression with HCK. NLRP1 and NLRP3 also showed
significant co-expression with HCK. This can be explained by the fact that co-expression is less
stringent than co-occurrence. In summary, NLRP12 is the only NLRP protein that shows co-
occurrence with HCK, and it also shows co-expression with HCK, although it is not the only
member of the NLRP family to do so. Other data sets, including Hausera data, Metzeler1 data,
Metzeler2 data, and Raponi data, were not available to evaluate the co-expression of NLRP12
versus HCK, as these other data sets did not incorporate data regarding NLRP12 expression.

Other potential binding partners of NLRP12 from the yeast two-hybrid screen

Other potentially interesting and unique genes that whose products may interact with NLRP12’s
PYD + NBD were identified in the yeast two-hybrid screen (Table 1 and Supplementary
materials, Table S1). Table 1 only shows those hits with frequency larger than 2, and these hits
were analyzed through an Ingenuity Pathway Analysis (IPA) diagram (Figure 8). In the IPA
diagram, protein-protein interactions found by our yeast two-hybrid screen are connected by
solid lines and interactions between protein binding partners collated by IPA are connected by
dashed lines. In addition, those molecules that are associated with AML are colored purple.
Interestingly, NLRP12 is also associated with AML from the database Catalogue of somatic
mutations in cancer (COSMIC). Although the mutation occurs in the NBD of NLRP12, only 2
samples of these mutations are observed within 175 samples and may be worthy of follow-up
study.
The interaction of one of the putative binding partners to NLRP12 identified in our screen, TRAF3 interacting protein 3 (TRAF3IP3), was validated in a co-immunoprecipitation experiment where the FLAG-tagged form of TRAF3IP3 and V5-tagged form of NLRP12 were overexpressed in 293T cells. TRAF3IP3 was coimmunoprecipitated by adding mouse monoclonal anti-TRAF3IP3 antibody, and a band that showed the same molecular weight size of NLRP12 was detected by rabbit monoclonal anti-V5 antibody (Figure 8). However, further validation of these other putative interactors detected by the yeast two-hybrid needs to be done.

**Discussion**

In this paper, the yeast two-hybrid assay was used to screen for NLRP12 binding partners. This approach identified 48 genes as positive hits encoding proteins that could possibly bind to NLRP12. Among the hits, the c-SRC non-receptor tyrosine kinase family member HCK was selected to focus upon as a potentially interesting target, particularly given its association with hematopoiesis and AML. Further direct screens found that NLRP12 specifically binds to HCK, but not to NLRP8 or NLRP3. Conversely, HCK, but not other members of the c-SRC non-receptor tyrosine kinase family, specifically binds to NLRP12. HCK’s C-terminal 30 amino acids are sufficient for binding to NLRP12. And, amino acids F503, I506, Q507, L510, and D511 of HCK are critical for binding to NLRP12. Co-immunoprecipitation experiments from mammalian cells confirmed that NLRP12 and HCK interact. Co-expression of NLRP12 and HCK results in a significantly lower steady-state expression level of NLRP12; thus, the interaction may affect NLRP12 protein stability. Finally, a bioinformatics analysis shows that *NLRP12* and *HCK* essentially exclusively co-occur, and mRNA levels for both are correlated in
NLRP12 binds to HCK

blood samples from AML patients. Together, these data suggested that NLRP12 and HCK
protein and mRNA co-expression may have an effect on the AML.

The binding data from yeast two-hybrid (Figure 3 and Figure 4) showed that the R2 and R3
regions are both important for binding, but we suspect that R3 region is the major region that is
critical for binding, while R2 region may be an “auxiliary” region that is also critical for binding,
because when we delete the R2 region but maintain the R3 region, the R3 region itself cannot
maintain stable binding of HCK’s C-terminal 40 amino acids to NLRP12’s PYD + NBD. Thus,
binding of NLRP12’s PYD + NBD to this fragment of HCK may occur initially through binding
to R3, followed by stabilization of the interaction by secondarily binding to R2. This model is
also consistent with the finding that when we mutated the R3 region, the binding of HCK’s C-
terminal 40 amino acid fragment to the NLRP12’s PYD + NBD was lost despite the R2 region
being present. The above evidences are consistent with the model that R2 and R3 regions
together are both necessary for binding, but the stabilization of binding to the R3 region may
require the presence of R2.

The yeast two-hybrid screen with NLRP12 also identified products from 47 other genes as
potential interactors. According to the Search Tool for Recurring Instances of Neighbouring
Genes (STRING) protein network and previous publications, NLRP12 interactors include
IRAK1 (39), NIK (35), TRAF3(3), heat shock protein 90 (HSP90) (40), Fas associated factor 1
(FAF1) (41), and apoptosis-associated speck-like protein containing a caspase activation and
recruitment domain (ASC) proteins. IRAK1, NIK, TRAF3, and Heat shock protein (HSP) 90
were only found in experiments involving semi-endogenous protein co-immunoprecipitations
with NLRP12. FAF1 was found to be an interacting protein in NMR experiments by showing a
difference in the chemical shift perturbation of FAF1’s ubiquitin-associated domains (UBA)
domain alone and NLRP12’s PYD domain bound to FAF1’s UBA domain. And FAF1 was also
found to interact with NLRP12 in a yeast two-hybrid study where the PYD domain of different
NLRP proteins were screened (42). However, in our study, IRAK1 (43), NIK (44), TRAF3 (45),
HSP90 (46), FAF1 (47), and ASC (48) proteins were not found in our results of interacting
proteins, although they are all expressed in leukocytes. Therefore, given these differences, the
results from this yeast two-hybrid screen may provide a unique database for future investigations
on the proteins that interact with NLRP12, and the functional outcomes of these interactions.

How HCK co-expression affects NLRP12 expression can be further investigated to determine the
functional outcomes of the NLRP12-HCK interaction relative to protein stability or degradation.
For example, MG-132 and chloroquine, which are a proteasomal inhibitor and an inhibitor of
lysosomal degradation, respectively, can be added to cells in these experiments to determine
whether the decreasing protein expression level of NLRP12 in the presence of HCK is due to
proteasomal degradation or lysosomal degradation. In addition, an NLRP12 stable cell line, with
a typically lower level of NLRP12 expression, compared to transiently transfected cells, can be
used to test formally whether NLRP12 protein expression levels are increasingly decreased when
the expression of HCK is increased. Moreover, the rate of NLRP12 degradation, in the absence
or presence of HCK, can be studied and compared in pulse-chase assays (35).

Given the interaction that was characterized between NLRP12 and HCK, we sought to identify
diseases in which their interaction may be relevant. AML is a type of blood and bone marrow
NLRP12 binds to HCK

cancer that is characterized by an unusually high level of circulating immature white blood cells (49). HCK is found to be highly expressed in hematopoietic stem cells of AML patients (24). The overexpression of HCK is linked with the development of AML (24,50). Therefore, HCK may be potentially used as a blood biomarker to identify those patients with potential to develop AML disease. In addition, activation of HCK and its signaling pathways often causes the development of AML (25,51). HCK has been reported to be involved in the feline McDonough sarcoma (fms)-like tyrosine kinase 3 (FLT3)-HCK- cyclin D-dependent kinase (CDK6) pathway. The mutation in FLT3-internal tandem duplications (FLT3-ITD) causes the activation of HCK, and subsequently results in overexpression of CDK6 in MV4-11 cells, a human AML cell line with the FLT3-ITD mutation. CDK6 siRNA resulted in MV4-11 cells with the FLT3-ITD mutation having a lower proliferation rate compared to MV4-11 control cells (25). Also, it has been reported that in AML patients, HCK, LYN, and FGR phosphorylation levels are high (51). In summary, the above evidence suggests that either high expression and/or high activation of HCK could occur in AML patients as well.

Our bioinformatics analysis revealed that NLRP12 is likely the only member of the NLRP family that shows significant co-occurrence with HCK at the level of mRNA expression. However, our cellular functional study revealed that NLRP12 protein expression levels decreased when it is co-expressed with HCK. This discrepancy might be due NLRP12 mRNA expression level not correlating with protein expression level (52-54). Nevertheless, these data are still consistent with the possibility that NLRP12 may have an effect on AML through its binding to HCK. NLRP12 and HCK co-expression and co-occurrence may then also be relevant to the prognosis
of AML. However, further analyses need to be performed to evaluate the ability of NLRP12 and HCK to serve as biomarkers in the prognosis of AML.

NLRP12 and HCK co-expression and co-occurrence may indicate that NLRP12 and HCK mRNA or protein expression can impact the same pathway or both be regulated by the same molecule. For example, in the case two molecules exhibiting co-occurrence, they may interact in the same signaling pathway, for example, Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and glycogen synthase kinase 3 beta (GSK3β) showed co-occurrence in the AML provisional data, and they are in the same signaling pathway (55).

Although many HCK inhibitors have been developed, they often lack specificity, with HCK inhibitors also inhibiting other members of the c-SRC non-receptor tyrosine kinase family (24,56-59). If the potential for the development of AML can be decreased when NLRP12 does not bind to HCK, then an inhibitor to the interaction between NLRP12 and HCK can be designed. On the other hand, if the potential for AML is increased when NLRP12 and HCK does not bind, then a drug that facilitates NLRP12 and HCK interaction could be developed. By this approach, the non-specificity problem with NLRP inhibitors may still exist but perhaps to a smaller extent, as NLRP proteins are less conserved overall compared to the members of the c-SRC non-receptor tyrosine kinase family. For example, the amino acid identity between NLRP12’s PYD + NBD versus another NLRP PYD + NBD most closely related to NLRP12, NLRP3’s PYD + NBD, is 54%. But, the protein identity between the HCK C1 fragment versus the one with the closest relationship, the LYN C1 fragment, is 78%.
In conclusion, this paper reports the preliminary findings of NLRP12 interacting with HCK, and NLRP12 co-occurs with HCK in AML patients. The binding data presented here will expand the database regarding the interactors of NLRP12, and additional bioinformatic and functional in vitro and in vivo analyses may shed light on the functional consequence of the interaction of NLRP12 with HCK.

Material and Methods

Reagents and cells

U937, THP-1, RAW 264.7, and 293T cells were all purchased from American Type Culture Collection (ATCC) (Manassas, VA). pCDNA-NLRP12 plasmid and pCDNA-NLRP3 plasmid were gifts from Dr. Jenny P. Y. Ting (University of North Carolina-Chapel Hill). pCDNA-NLRP8 plasmid was purchased from Genescript (Piscataway, NJ). All other chemicals were reagent grade. The mouse monoclonal anti-FLAG antibody and the rabbit monoclonal anti-FLAG antibody were purchased from Sigma-Aldrich (St. Louis, MO). The mouse monoclonal anti-HCK antibody, the mouse monoclonal anti-TRAF3IP3 antibody, and the mouse IgGκ binding protein-horseradish peroxidase (HRP) for detecting the anti-HCK antibody were purchased from Santa Cruz Biotechnology (Dallas, TX). The rabbit monoclonal anti-V5 antibody and polyclonal rabbit HRP-conjugated anti-mouse IgG secondary antibodies were purchased from Cell Signaling (Beverly, MA). The goat anti-rabbit IgG HRP-linked secondary antibody was purchased from Cell Signaling. The Clean-Blot™ IP Detection Reagent was purchased from Thermo Fisher Scientific (Grant Island, NY). Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 488 and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555 were purchased from Thermo
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Fisher Scientific (Grant Island, NY). The 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from Thermo Fisher Scientific.

**Cloning**

*NLRP12*’s PYD + NBD (corresponding to a.a. 1-531) was amplified by polymerase chain reaction (PCR) (primers see Table 3), from a pCDNA-*NLRP12* plasmid, was digested with enzymes NdeI and SalI, and subcloned into the pGBKT7 vector (Takara). The sequence encoding the PYD + NBD of *NLRP3* (corresponding to a.a. 1-536) was amplified by PCR (see table 3 for primers), digested with enzymes NdeI and BamHI, and subcloned in the pGBKT7 vector. The sequence encoding the PYD + NBD of *NLRP8* (corresponding to a.a. 1-527) was amplified by PCR (see Table 3 for primers), digested with enzymes EcoRI and BamHI, and subcloned into the pGBKT7 vector. The entire sequences corresponding to the C-terminal 42 amino acids of FGR, c-SRC, YES, and FYN, and 40 amino acids of BLK, LCK, LYN, and HCK (The C terminal 40 (or 42) amino acids is called “C1”), and HCK constructs C2, C3, C4, C5, C6, C7, N1, N2, N3, N4, N5, M1, M2, M3, M4, and HCK C1 mutations for alanine scanning with F503A, E504A, Y505A, I506A, Q507A, S508A, V509A, L510A, and D511A, were all synthesized from IDT DNA (Coralville, IA) and cloned into the pACT2 vector (Takara).

*NLRP12* was amplified by PCR (see Table 3 for primers), digested with AflII and XbaI, and subcloned into pEFIRES-P-3FLAG (60) and pEFIRES-P-V5 (60). 3FLAG and V5 are homemade, i.e., added later into pEFIRES-P. The peptide sequence of the 3FLAG epitope is DYKDDDDKDYKDDDDK. The peptide sequence of the V5 epitope is GKPIPPLLGLDST. The multiple cloning sites of pEFIRES-P were modified according to our needs. Sequences encoding HCK p61 and p59 isoforms were amplified by PCR (see Table 3 for
primers), digested with AflIII and XbaI, and subcloned into pEFRES -3FLAG vector. Final plasmids were sequenced to confirm the constructs.

Yeast two-hybrid assay

Yeast transformed with the plasmid PGBK7- NLRP12’s PYD + NBD

Yeast strain AH109 (Takara, Mountain View, CA) was stored frozen at -80°C. Upon use, it was streaked onto an agar plate supplemented with yeast extract, peptone, dextrose, and adenine hemisulfate (YPDA) (Takara), which was then placed in a 30°C incubator overnight. The growing colonies were picked and placed into 10 ml of YPDA media, incubated overnight with shaking at 30°C. Then they were transformed with NLRP12’s PYD + NBD plasmid, following recommendations in the Takara manual (Matchmaker™ Gal4 Two-hybrid System 3 and Libraries User Manual). A 4ml of 1 M of 3-AT was added into 800 mL of the YPDA medium that were used for making the plates due to a leakage problem. 3-AT is a competitive inhibitor for the product of the HIS3 reporter gene. Yeast cells were lysed using a trichloroacetic acid (TCA) method, and western blots were performed to check NLRP12’s PYD + NBD protein expression (data not shown).

Library scale transformation of cDNA library into yeast that have been transformed with the PGBK7- NLRP12 PYD + NBD plasmid

A human leukocyte Matchmaker™ cDNA library was purchased from Takara. The library was titered according to the Matchmaker™ Gal4 Two-hybrid System 3 and Libraries User Manual. The colony-forming units (CFU) for the library were calculated to be about 3.1 x 10^9 CFU/ml. Four large scale transformations (equal to one library scale) were done at the same time.
following the Matchmaker™ Gal4 Two-hybrid System 3 and Libraries User’s Manual. To calculate the efficiency of yeast two-hybrid (i.e., the number of colons obtained per µg of library plasmid DNA) and to check the expressions of “bait” and “prey” plasmids, small amounts of the solution, diluted 10X, 100X, 1000X, and 10,000X were plated in SD/-Trp-Leu 2DO (drop out) plates. The yeast two-hybrid efficiency obtained was 3 x 10^6 CFU per µg of library plasmid DNA. In addition, if the yeast grew on the SD/-Trp-Leu-His-Ade + 3-AT plates, the “bait” and “prey” plasmids were considered to be interacting with each other.

*Small scale transformation*

The yeast two-hybrid co-transformation on a small scale for all of the direct screenings was performed following the small-scale transformation protocol in the Matchmaker™ Gal4 Two-hybrid System 3 & Libraries User’s Manual. The direct screens were to test whether NLRP12 PYD + NBD and HCK specifically interact; identification of HCK F503, Q507, L510, and D511 being critical for binding with NLRP12 PYD + NBD; and the characterization of the HCK C-terminal 30 amino acid fragment binding to NLRP12 PYD + NBD.

*Cell transfection*

293T cells, THP-1 cells, U937 cells, and RAW 264.7 cells were seeded at a density of 5 x 10^5 per well in a 6-well plate. Transfection followed the Lipofectamine® 3000 reagent (Thermo Fisher Scientific) protocol with the following modifications. Briefly, 3.75 µl of Lipofectamine® 3000 was added into 125 µl of Opti-MEM® medium (Thermo Fisher Scientific). 2.5 µg of plasmid DNA (prepared by isolation through cesium chloride (CsCl) density gradient purification (61) and 5 µl of P3000™ reagent were added into another aliquot of 125 µl of Opti-
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MEM® medium. Then these two tubes were mixed together, and the mixed tube was incubated at room temperature for 15 min. 250 µl of this mixture was added to the cells. The cells were incubated at 37 °C with 5% CO₂ for two days until analysis.

**NLRP12 stable cell lines**

THP-1 cells, U937 cells, and RAW 264.7 cells were transfected with pEFires-P-NLRP12-3FLAG plasmid, and 2.5 µg/ml puromycin (Invitrogen™ by Thermo Fisher Scientific) was added. After two weeks, the cells were considered to be stably expressing NLRP12 after screening for expression. The stable cells were “pooled” clones, and they are maintained by adding 2.5 µg/ml puromycin.

**Cell lysis and immunoprecipitation**

After trypsinization, cells were lysed in 500 µl of lysis buffer (1% NP-40, 4mM Tris-HCl, pH 8.0, 150 mM NaCl). Cells were then treated with freeze-thaw cycles, i.e., frozen in the -80°C freezer for 5 min and thawed at 37°C in a water bath for 3 min, for a total of five times. Cells were then centrifuged at 12,000 x g for 10 min at 4°C. After centrifugation, the supernatant was saved at -20°C until further use.

For immunoprecipitations, cell lysates were pre-cleared by adding 40 µl of protein A/G agarose beads (Thermo Fisher Scientific) from a 50% slurry, and the sample was rotated for 1 hr at 4°C. The sample was spun at 1000 x g for 1 min. Then, 30 µl of pre-cleared cell lysate were removed and run on immunoblotting as a control. The remaining cell lysate was incubated with 1 µg anti-HCK mouse monoclonal primary antibody overnight. On the second day, 30 µl of protein A/G
agarose, from a 50% slurry and previously washed with lysis buffer three times, were added to the tube, and the tube was incubated for 3 hrs at 4°C. The beads were then washed with lysis buffer, but with the NaCl concentration increased to 500 mM. The tube was briefly centrifuged at 1000 x g for 1 min., and the supernatant was removed. This step was performed for a total of 5 times. The proteins were eluted from the beads by addition of SDS-PAGE sample buffer.

**Immunoblotting**

Protein concentrations were determined by the Pierce™ BCA protein assay (Thermo Fisher Scientific). About 20 µg of cell lysate samples and immunoprecipitated samples were loaded onto the 8% acrylamide gel. After overnight transfer of the proteins from an SDS-gel to a 0.45 μm pore size polyvinylidene difluoride (PVDF) membrane, the membrane was blocked in for 1 hr at room temperature 5% non-fat dry milk dissolved in (Tris-buffered saline with Tween 20, pH=8.0) (TBST) (Sigma-Aldrich). Mouse monoclonal anti-FLAG antibody (diluted 1:1000) and mouse monoclonal anti-HCK antibody (diluted 1:1000) were added and the membrane incubated overnight. For detection of bands in the cell lysate, rabbit anti-mouse IgG HRP-linked secondary antibody (diluted 1:2000) (for anti-FLAG antibody) and mouse IgGκ binding protein-HRP secondary antibody (for anti-HCK antibody) (diluted 1:1000) were added, and the membrane was incubated for 1 hr at room temperature. For immunoprecipitations, Clean-Blot™ IP detection reagent (HRP) secondary antibody (1:300) (Thermo Fisher Scientific) was used. The detection signal was visualized by using enhanced chemiluminescence (ECL) reagent (Thomas Scientific, Swedesboro, NJ). Immoblots were analyzed by chemiluminescence on a Bio-Rad ChemiDoc™ Touch Gel imaging system (Bio-Rad, Hercules, CA).
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**Immunofluorescence**

The coverslips were autoclaved and coated with 0.1 mg/ml of poly-D-lysine hydrobromide (mol wt 70,000-150,000) (Sigma-Aldrich). Cells were plated onto the coverslips. The cells were fixed in 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) in phosphate buffered saline (PBS) for 15 mins. And fixation was stopped by adding 50 mM NH₄Cl in PBS for 15 min at room temperature. After raising with PBS, the fixed cells were permeabilized by adding 0.5% Triton®-X-100 in PBS for 10 min. The cells were then washed with PBS three times and blocked with 0.1% of BSA in PBS at room temperature for 1 hr. The rabbit monoclonal anti-FLAG antibody (diluted 1:100) and mouse monoclonal anti-HCK antibody (diluted 1:100) were added to each cover slip and incubated overnight. Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 488 (diluted 1:100) and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555 (1: 100) were added. After incubation with secondary antibodies for 1 hr, the cells were washed five times with PBS. During the first step, DAPI (Invitrogen™ by Thermo Fisher Scientific) was also added at 1:1000 in PBS for 2 min. Finally, the coverslips were mounted in ProLong™ Gold Antifade mounting medium (Life Technologies Corporation by Thermo Fisher Scientific, Eugene, OR). The stained slides were viewed on a ZEISS LSM 880 (Pleasanton, CA) with Airyscan. The images were viewed and processed using Fiji software (62).

**IPA diagram**

Yeast two-hybrid data were analyzed by IPA software (QIAGEN Inc.,

Statistical analysis

The Spearman coefficient and Pearson coefficient used for co-expression was generated by the cBioPortal website (37,38,63). Clinical samples from the Oncomine website (https://www.oncomine.org) were downloaded and checked for NLRP12 and Hck mRNA expression levels. The Fisher exact test and the odds ratio test used for co-occurrence were generated by the cBioPortal website. Analysis of variance (ANOVA) was performed to check whether a significant difference of the treatment exists among all the groups. A p-value < 0.05 was considered statistically significant. If P < 0.05, then the Tukey test was used to determine whether a pair-wise difference exists. Quantification of the western blot bands was performed by FIJI software (62) and made into graphs by Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Acknowledgement

Thank you to Dr. Houda Alachkar for providing valuable suggestions to this paper. We also thank Ms. Meng Li and Dr. Yibu Chen at the University of Southern California’s Norris Medical Library for their suggestions for bioinformatic analysis. Appreciation is given to Mr. Noam Morningstar-Kywi and Dr. Ian Haworth for their helps on structural analysis.

Abbreviations

3-AT: 3-Amino-1,2,4-triazole; AML: acute myeloid leukemia; ANOVA: analysis of variance; ASC: apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; ATCC: American Type Culture Collection; C1: self-named truncations of C-terminal 40 (or 41) amino acids fragment of c-SRC family non-receptor tyrosine kinases; C2-
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C7 and N1-N5: self-named truncations HCK C1 fragment; CD40: cluster of differentiation 40;
CDK6: cyclin D-dependent kinase; CFU: colony-forming units; COSMIC: catalogue of somatic
mutations in cancer; CsCl: cesium chloride; DAMP: damage-associated molecular patterns;
DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride; DC: dendritic cells; DO: drop out (2DO:
SD/-Trp-Leu; 4DO: SD/-Trp-Leu-His-Ade); ECL: enhanced chemiluminescence; FAf1: Fas
associated factor 1; FLT3-ITD: FLT3-internal tandem duplications; FLT3: feline McDonough
sarcoma (fms)-like tyrosine kinase 3; GSK3b: glycogen synthase kinase 3 beta; HCK C1
fragment: HCK C-terminal 40 amino acids; HCK: hematopoiesis cell kinase; HRP: horseradish
peroxidase; HSP90: heat shock protein 90; IL: interleukin; IPA: ingenuity pathway analysis;
IRAK1: IL-1 receptor-associated kinase 1; KO: knock out; KRAS: Ki-ras2 Kirsten rat sarcoma
viral oncogene homolog; LRR: leucine rich repeat domain; M1: the amino acids in the R1 region
were mutated to the amino acids identical to the R1 region in Lyn; M2: the amino acids in the
R2 region were all mutated to Alanine; M3: the amino acids in the R3 region were all mutated to
Alanine; M4: the amino acids in the R4 region were all mutated to Alanine; NBD: nucleotide
binding domain; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NIK:
NF-κB-inducing kinase; NLR: nucleotide binding domain and leucine-rich repeat; NLRP: NLR
pyrin domain-containing protein; PAMP: pathogen-associated molecular patterns; PBS:
phosphate buffered saline; PCR: polymerase chain reaction; PDB: protein data bank; PMA:
phorbol 12-myristate 13-acetate; PRR: pattern recognition receptors; PVDF: polyvinylidene
difluoride; PYD: pyrin domain; R1, R2, R3, and R4: self-named four regions in the HCK C
terminal 40 amino acids fragment; SD: synthetic dropout; SD/-Trp-Leu: SD base plus amino
acids supplements minus tryptophan and leucine; SD/-Trp-Leu-His-Ade: SD base plus amino
acids supplements minus tryptophan, leucine, and histidine, and adenine; STRING: search tool
NLRP12 binds to HCK for recurring instances of neighboring genes; TBST: tris-buffered saline with Tween 20, pH=8.0; TCA: trichloroacetic acid; TCGA: the cancer genome atlas; TLR: toll-like receptor; TRAF3: tumor necrosis factor receptor associated factor 3; TRAF3IP3: TRAF3 interacting protein 3; UBA: ubiquitin-associated domains; WT: wild type; YPDA: yeast extract, peptone, dextrose, and adenine hemisulphate.

Competing Interest

The authors have declared that no competing conflict exists.

Author Contributions Statement

YZ design the experiment and wrote and revised the manuscript. CO helped to design the experiments and to revise the manuscript.

References

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60. Hobbs S, Jitrapakdee S, Wallace JC. Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1α promoter for creation of stable mammalian
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cell lines that express very high levels of recombinant proteins. Biochem Biophys Res Commun. 1998; 252: 368-372.


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<td>Deleted in azoospermia (DAZ) associated protein 2 (DAZAP2)</td>
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<td>Filamin A, alpha (FLNA)</td>
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<tr>
<td>Hematopoietic cell kinase (HCK) proto-oncogene, c-SRC family tyrosine kinase (HCK)</td>
<td>9</td>
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<tr>
<td>Chaperone DnaJ (a.k.a., heat shock protein 40 kD(Hsp40)) homolog, subfamily B, member 1 (DNAJB1)</td>
<td>9</td>
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<tr>
<td>Metallothionein 2A (MT2A)</td>
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<td>Cluster of differentiation 14 molecule (CD14)</td>
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<td>DEAD (Asp-Glu-Ala-Asp) box helicase 5 (DDX5)</td>
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<td>Tumor necrosis factor (TNF) receptor associated factor 3 (TRAF3) interacting protein 3</td>
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(TRA3FIP3)

| Thyroid hormone receptor interactor 4 (TRIP4) | 3 |

Table 2: Co-occurrence of mRNA expression level of HCK and all of the NLRP family proteins (from the provisional data, assessed on July 11 2019)

<table>
<thead>
<tr>
<th>HCK + NLRP family genes</th>
<th>Co-occurrence (C) or mutual exclusivity (M)</th>
<th>P-value</th>
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<tr>
<td>NLRP1</td>
<td>C</td>
<td>0.048</td>
</tr>
<tr>
<td>NLRP2</td>
<td>M</td>
<td>0.546</td>
</tr>
<tr>
<td>NLRP3</td>
<td>C</td>
<td>0.415</td>
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<tr>
<td>NLRP4</td>
<td>M</td>
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<td>NLRP5</td>
<td>M</td>
<td>0.877</td>
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<td>NLRP6</td>
<td>M</td>
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<td>NLRP7</td>
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<td>NLRP8</td>
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<td>NLRP9</td>
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<td>NLRP12</td>
<td>C</td>
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</tr>
<tr>
<td>NLRP13</td>
<td>C</td>
<td>1.000</td>
</tr>
<tr>
<td>NLRP14</td>
<td>M</td>
<td>0.509</td>
</tr>
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Table 3: Primers used in cloning (The underline shows the restriction enzymes)
### NLRP12 binds to HCK

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<th>Name</th>
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<tr>
<td><strong>NLRP12</strong> 5’ primer</td>
<td>GATCTACA<strong>CATATG</strong>CTACGAACCGACGGCA</td>
</tr>
<tr>
<td>(cloned into PGBK7)</td>
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</tr>
<tr>
<td><strong>NLRP12</strong> 3’ primer</td>
<td>GATGTAG<strong>TCGACT</strong>CATACCCTCGTCCAGGATATAGTACATAGCT</td>
</tr>
<tr>
<td>(cloned into PGBK7)</td>
<td></td>
</tr>
<tr>
<td><strong>NLRP3</strong> 5’ primer (cloned into pGBK7)</td>
<td>GATCTAC<strong>ATACTAG</strong>GAAGATGGCAAGCACCCGCCTGCA</td>
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<tr>
<td><strong>NLRP3</strong> 3’ primer (cloned into PGBK7)</td>
<td>GATGTAGG<strong>ATTCCTCAG</strong>AGGTAGATCATGGGGCAAAGA</td>
</tr>
<tr>
<td><strong>NLRP8</strong> 5’ primer (cloned into pGBK7)</td>
<td>GATCTAG<strong>AATTCTAG</strong>GTACGTGAATCCACCCTCTG</td>
</tr>
<tr>
<td><strong>NLRP8</strong> 3’ primer (cloned into PGBK7)</td>
<td>GATGTAGG<strong>ATTCCTCAG</strong>AGGTCTTTGTGGGAAACAGAGAAAC</td>
</tr>
<tr>
<td><strong>NLRP12</strong> 5’ (cloned into pEFires-P-3FLAG and pIRES-V5)</td>
<td>G<strong>TCCAGCTTAAG</strong>GTACGAACCGACGGCA</td>
</tr>
<tr>
<td><strong>NLRP12</strong> 3’ (cloned into PIRES-3FLAG and pEFires-P-V5)</td>
<td><strong>GTTCAATCTTAG</strong>GCAGCCAATGTCCAAATAAGG</td>
</tr>
<tr>
<td><strong>HCK</strong> p61 5’ (cloned into pEFires-P-3FLAG)</td>
<td>GATCTACT<strong>TAAGGCC</strong>ACCATGGGGGGGCGCTCAAGCTGGGCGG</td>
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<tr>
<td><strong>HCK</strong> p59 5’ (cloned into pEFires-P-3FLAG)</td>
<td>GATCTACT<strong>CGAGGCC</strong>ACCATGGGGTGCATGAAGTCCAAAGTTCC</td>
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**Figure legends:**

**Figure 1:** (A) Schematic drawing of the domain structure of full-length NLRP12 (top) and the domains used as the construct for the yeast two-hybrid screen (bottom). PYD: Pyrin domain; NBD: Nucleotide binding domain; and LRR: Leucine rich repeat domain. (B) The domain structures of the two alternatively spliced isoforms of HCK, HCK p59 and HCK p61. (C) Identification of the clones of HCK interacting with NLRP12 ("hits") obtained from the yeast two-hybrid screen. Among the nine of the clones positive for HCK, one clone encoded for the smallest interacting fragment of HCK, the C-terminal 40 amino acids of HCK. The numbers in parentheses indicate the total number of hits obtained in the screen.

**Figure 2:** Specificity of binding of NLRP12 with HCK. (A) Schematic drawing of HCK (p61 isoform, top) and, for comparison, the region of HCK ("HCK C1 fragment," lower) used for testing the specificity of binding of HCK to other members of the NLRP family of proteins. The numbers represent the amino acid residues of the protein or protein fragment (B) Phylogenetic tree showing the relationships among the PYD + NBD region of all of the members of the human NLRP family of proteins. The phylogenetic tree was produced through Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). For each NLRP family member, the PYD + NBD of the longest isoform was selected for generating the phylogenetic tree. NLRP3 is phylogenetically the closest NLRP to NLRP12, while NLRP8 is phylogenetically among the most distant from NLRP12. NLRP3 and NLRP8 are highlighted in red color. (C) When
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NLRP12’s PYD + NBD and HCK C1 fragment were co-transformed into yeast, they grew on the highest stringency plates (4DO + 3AT), consistent with an interaction between the two constructs. On the other hand, neither NLRP3’s PYD + NBD co-transformed with the C1 fragment nor NLRP8’s PYD + NBD co-transformed the C1 fragment grew on the highest stringency plates. These results are consistent with a specific interaction between NLRP12 and the HCK C1 fragment, but not with the other NLRPs. (D) Yeast clones grew on the high stringency plates 4DO + 3AT when HCK’s C1 fragment and NLRP12’s PYD + NBD were co-transformed, but they did not grow on the high stringency 4DO + 3AT plates when the C1 fragments of other members of the c-SRC family of non-receptor tyrosine kinases were co-transformed with NLRP12’s PYD + NBD. These results are again consistent with a specific interaction between NLRP12 and HCK.

Figure 3: (A) Structure of the HCK C1 fragment generated by Chimera from the University of California, San Francisco (version 1.13.1), extracted from its structure within the entire HCK protein. HCK C1 fragment was thus divided into four helical domains: R1, R2, R3, and R4. The side chains of amino acids F503, I506, Q507, L510, and D511 are shown. However, it is not clear whether the C1 fragment retains the structural features shown here when it expressed on its own. (B) Primary amino acid sequence alignments showing of the C1 fragments of all members of the c-SRC non-receptor tyrosine kinase family (40 amino acids for HCK, BLK, LCK, and LYN; 42 amino acids for FGR, c-SRC, YES, and FYN). The black lines indicate the R1, R2, R3, or R4 regions. The arrows denote the four critical amino acids for binding of HCK C1 fragment to NLRP12’s PYD + NBD. (C) Phylogenetic tree showing the relationship among all of the C1 fragments of the members of the c-SRC family of non-receptor tyrosine kinases. The
phylogenetic tree was produced through Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). HCK is highlighted in red color. (D) Four sets of distinct mutations were made within the HCK C1 fragment. M1: the amino acids in the R1 region were mutated to the amino acids identical to the R1 region in LYN (highlighted in red color); M2: the amino acids in the R2 region were all mutated to alanine (Ala); M3: the amino acids in the R3 region were all mutated to Ala; and M4: the amino acids in the R4 region were all mutated to Ala. (E) Yeast did not grow on high stringency 4DO + 3AT plates when HCK C1 fragment with the M3 mutations was co-transformed with NLRP12’s PYD + NBD. But yeast can grow on the high stringency 4DO + 3AT plates when the HCK C1 fragment contained either the M1, M2, or M4 mutations, suggesting that the R3 region within the C1 fragment was necessary for binding to NLRP12 NBD + PYD. (E) Yeast did not grow on high stringency 4DO plates when NLRP12’s PYD + NBD was co-transformed with HCK C1 fragment with single Ala substitutions at either F503, I506, Q507, L510, or D511. But yeast grew on high stringency 4DO plates when NLRP12’s PYD + NBD was co-transformed with HCK’s C1 fragment in which the following amino acids in the R3 region were individually mutated to Ala: E504, Y505, S508, or V509.

Figure 4: (A) Top: schematic drawing of HCK truncations comprising the C1 to C7 fragments. Bottom: yeast grew on the high stringency 4DO plates and 4DO +3AT plates when HCK’s C-terminal 40 amino acid fragment (C1), HCK’s C-terminal 35 amino acid fragment (C2), and HCK’s C-terminal 30 amino acid fragment (C3), individually, were co-transformed with NLRP12’s PYD + NBD. But yeast did not grow on the high stringency 4DO plates nor 4DO+3AT plates when C4 to C7 fragments were co-transformed to with NLRP12’s PYD + NBD. The lack of yeast growth upon co-transformation of the C7 fragment and NLRP12 are not shown. (B)
Top: schematic drawing of Hck truncations comprising the N1 to N5 fragments. Bottom: yeast
grew on the high stringency 4DO plates and 4DO +3AT plates when *HCK*'s C-terminal 40
*amino acid fragment (C1)* was co-transformed with *NLRP12’s PYD + NBD (control).* But yeast
did not grow on the high stringency 4DO plates nor 4DO+ 3AT plates when *N1 and N5*
*fragments* were co-transformed with *NLRP12’s PYD + NBD,* suggesting that the very C-terminal
5 amino acids are also necessary for binding of NLRP12. The colored boxes represent different
fragments that were deleted each time.

**Figure 5:** NLRP12 co-immunoprecipitates with HCK and co-localizes with HCK by
immunofluorescence. (A) NLRP12 co-immunoprecipitated with HCK when both proteins were
transiently exogenously co-expressed in 293T cells, with NLRP12 having to be expressed as
epitope-tagged forms. Cells were lysed using a non-denaturing cell lysis buffer (see Material
and Methods). Co-immunoprecipitations were done using a mouse monoclonal anti-HCK
antibody followed by protein A/G agarose. The immunoprecipitated proteins were analyzed by
immunoblotting. The same method was used for experiments shown in (B), (C), and (D). (B)
NLRP12 co-immunoprecipitated with endogenous HCK in macrophage-like RAW 264.7 cells,
when NLRP12 is exogenously and stably expressed. Arrows show the two isoforms of HCK p59
and p61 that were immunoprecipitated by the anti-HCK antibody, although it is not clear
whether NLRP12 co-immunoprecipitated with one form or the other, or both. (C) Exogenously
and stably expressed NLRP12 co-immunoprecipitated with endogenously expressed HCK in
monocyte-like THP-1 cells. In these cells, phorbol 12-myristate 13-acetate (PMA) (1µM) was
added to enhance the expression of NLRP12 under the PMA-sensitive cytomegalovirus promoter
of the *NLRP12* plasmid. (D) Left panel shows that NLRP12 was exogenously and stably
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expressed in lymphoblast-like U937 cells. Right panel shows that NLRP12 co-
immunoprecipitated with endogenously expressed HCK in U937 cells after PMA was added to
1µM, as was done for THP-1 cells, to enhance expression of NLRP12. (E) Immunofluorescent
images showing the colocalization of a cohort of NLRP12 with either HCK p59 or p61. HCK
was immunolabeled with a mouse monoclonal anti-HCK antibody, followed by a secondary goat
anti-mouse IgG (H+L) cross-adsorbed, conjugated to Alexa Fluor 488. For NLRP12, the
primary antibody was a rabbit monoclonal anti-FLAG antibody, and the secondary antibody was
a goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555.
Nuclei were counterstained by DAPI.

**Figure 6:** The co-expression of HCK protein significantly decreased the steady-state protein
expression levels of NLRP12 (A) Epitope-tagged NLRP12-V5 and HCK p61 were co-
transfected into 293T cells. Cell lysates were immunoblotted with rabbit monoclonal anti-V5
antibody for NLRP12 and mouse monoclonal anti-HCK antibody. Comparing lane 2 (basal level
of NLRP12 expression) with lanes 5, 6, and 7, co-expression of HCK protein leads to a
significant decrease of protein expression levels of NLRP12 over three times they are co-
expressed. Increasing protein expression of HCK, relative to the plasmid DNA added (ramp:
lanes 5, 6, and 7; 2.5-20 µg of DNA) was not observed possibly because the amount of plasmid
for HCK transfection were already too high, resulting in the maximum expression of HCK at all
plasmid DNA amounts. The figure is representative of the results from three experiments. (B)
Quantification of the NLRP12 protein bands (mean + standard error) to show that NLRP12
protein expression levels decreased in the presence of co-expressed HCK, relative to equal
amounts of cell lysate being assayed. The NLRP12 protein expression levels are indicated as
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arbitrary units. (C) Quantification of the HCK protein bands (mean + standard error) confirming that HCK protein expression levels did not change although the amount of plasmid DNA for HCK increased. Figure (B) and (C) were generated by Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Twenty μg of cell lysate proteins were loaded for each lane. ANOVA and Tukey tests were used to compare the NLRP12 and HCK protein bands.

**Figure 7:** NLRP12 is co-expressed with Hck in acute myeloid leukemia (AML) patient samples. Both NLRP12 expression and Hck expression data are shown as log2 values. The data are RNA seq data obtained from cBioportal (provisional) database assessed on July 11, 2019. The graph was taken from the cBioportal website. Spearman coefficient, indicating the association of NLRP12 and Hck mRNA expression level (if NLRP12 and Hck mRNA expression levels are non-parametric) = 0.80. Pearson coefficient, indicating the degree of the linear relationship between the NLRP12 mRNA expression level and Hck mRNA expression level (if NLRP12 and Hck mRNA expression levels are parametric) = 0.73. The p-values for Spearman coefficient and Pearson coefficient are both < 0.001.

**Figure 8:** (A) Ingenuity Pathway Analysis (IPA) displaying the 13 distinctive hits from the total results of the yeast two-hybrid screen for proteins that interacted with NLRP12. The figure only shows the NLRP12-interacting proteins with hits that have a frequency larger than 2. The solid lines indicate interacting proteins found through our yeast two-hybrid screen. The dashed lines are the NLRP12-interacting proteins previously reported in published data. The purple-colored molecules are those associated with AML. (B) To validate an interaction shown in the IPA diagram, tumor necrosis factor receptor-associated factor 3 interacting protein 3 (TRAF3IP3)-
3FLAG and NLRP12-V5 were co-immunoprecipitated from co-transfected 293T cells. A non-denaturing cell lysate buffer (see Material and Methods) was used to lyse the cell, and co-immunoprecipitations were performed with a mouse monoclonal anti-TRAF3IP3 antibody followed by protein A/G agarose. The immunoprecipitates were analyzed for NLRP12 by immunoblotting with rabbit monoclonal anti-V5 antibody. The results from the co-immunoprecipitation are consistent with a confirmation of an interaction between TRAF3IP3 and NLRP12.
NLRP12 binds to HCK

Figure 1
NLRP12 binds to HCK

Figure 2
NLRP12 binds to HCK

1033
1034
1035
1036
NLRP12 binds to HCK

Figure 3
NLRP12 binds to HCK
Figure 4
NLRP12 binds to HCK

Figure 5
NLRP12 binds to HCK

Figure 6

(A) NLRP12-V5
HCK p61
NLRP12-V5
HCK
Lane 1 2 3 4 5 6 7

(B) NLRP12 protein expression levels

(C) HCK protein expression levels

HCK only 2.5 μg 2.5 μg 5 μg 10 μg 20 μg
NLRP12 binds to HCK

Figure 7

Spearman coefficient = 0.80
(p value < 0.001)

Pearson coefficient = 0.73
(p value < 0.001)
NLRP12 binds to HCK

Figure 8
Nucleotide binding domain and leucine-rich repeat pyrin domain-containing protein 12: characterization of its binding to hematopoietic cell kinase

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Running title: Nlrp12 binds to Hck

Key words: yeast two-hybrid, innate immunity, protein-protein interaction, Nlrp12, Hck, acute myeloid leukemia

Abstract
Protein-protein interactions are key to defining the function of nucleotide binding domain (NBD) and leucine-rich repeat (LRR), pyrin domain (PYD)-containing family of proteins 12 (Nlrp12). cDNA encoding the human PYD + NBD of Nlrp12 was used as bait in a yeast two-hybrid screen
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with a human leukocyte cDNA library as prey. Hematopoiesis cell kinase (Hck), a member of
the Src family of non-receptor tyrosine kinases, was among the top hits. The C-terminal 40
amino acids of Hck specifically bound to Nlrp12’s PYD + NBD, but not to that of Nlrp3 and
Nlrp8. Amino acids F503, Q507, L510, and D511 of Hck are critical for the binding of Hck’s C-
terminal 40 amino acids to Nlrp12’s PYD + NBD. Additionally, the C-terminal 30 amino acids
of Hck are sufficient to bind to Nlrp12’s PYD + NBD, but not to its PYD alone nor to its NBD
alone. In cell lines that express Hck endogenously, it was co-immunoprecipitated with stably
expressed exogenous Nlrp12. Also, Nlrp12 co-immunoprecipitated and co-localized with Hck
when both were overexpressed in 293T cells. In addition, in this overexpression system, steady-
state Nlrp12 protein expression levels significantly decreased when Hck was co-expressed.
Bioinformatic analysis showed that Hck mRNA co-occurred with Nlrp12 mRNA, but not with
other Nlrp proteins, in blood and marrow samples from acute myeloid leukemia (AML) patients.
The mRNA of Nlrp12 is also co-expressed with Hck in AML patient samples, and the levels of
mRNA expression of each are correlated. Together these data suggest that Nlrp12, through its
binding to Hck, may have an effect on the progression of AML.

Introduction

Pattern recognition receptors (PRR) are the first major line of cellular defense against damage-
associated molecular patterns (DAMP) and pathogen-associated molecular patterns
(PAMP)(Takeuchi and Akira, 2010). Among PRR, one class of proteins is the nucleotide-
binding domain (NBD) and leucine-rich repeat (LRR) receptor family (NLR). NBD and LRR
PYD protein 12 (Nlrp12) is one protein that belongs to the PYD subfamily of the NLR class and
is a multi-functional protein (Tuncer et al., 2014). Nlrp12’s functions likely all depend on
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defined protein-protein interactions. For example, in anti-inflammatory functions of Nlrp12, Nlrp12 interacts with tumor necrosis factor receptor associated factor 3 (TRAF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-inducing kinase (NIK) in the non-canonical NF-κB pathway, when toll-like receptors (TLR) are stimulated with Pam3Cys4 triacylated lipopeptides followed by addition of cluster of differentiation 40 (CD40) (Allen et al., 2012). Nlrp12’s inhibition of the non-canonical NF-κB pathway, thereby effecting an anti-inflammatory function, has been reported in many publications (7-11). For example, in colitis-driven colon cancer, Nlrp12 knock-out (KO) mice experience a higher frequency of death, weigh less, have a shorter colon length, and have more severe disease progression than wild type (WT) mice, all of which may occur through Nlrp12’s inhibition of the non-canonical NF-κB pathway (Zaki et al., 2011; Allen et al., 2012). In addition, Nlrp12 KO mice have been reported to exhibit higher innate immunity when it is infected by the virus because Nlrp12 inhibits the non-canonical NF-κB pathway; thus, in a KO mice, a more significant increase of NF-κB activity is observed compared to WT mice when it is infected by the virus (Chen et al., 2019). And in the case of Nlrp12 protecting the diversity of intestinal microbiota (Chen et al., 2017), thereby preventing obesity (Truax et al., 2018), it has also been reported that Nlrp12 KO mice display significantly decreased intestinal microbial diversity due to increased innate immunity, resulting from the lack of inhibition of the non-canonical NF-κB pathway.

In addition, Nlrp12 also interacts with interleukin (IL)-1 receptor-associated kinase 1 (IRAK1) in the canonical NF-κB pathway when TLRs are stimulated, also to inhibit the inflammatory response (Ye et al., 2008). In contrast, for human studies of Nlrp12-associated autoinflammatory disorders, no protein-protein interactions have been reported (Jeru et al., 2008; Jeru et al., 2011).
Functionally, however, Nlrp12 KO mice had a higher immunological response in the autoimmune model of contact hypersensitivity (Arthur et al., 2010); thus, these mice are more prone to develop contact hypersensitivity. This results most likely from Nlrp12 KO mice having defective migration of their dendritic cells (DC). But, in the mouse study, no protein-protein interactions had been reported either (Arthur et al., 2010). And Nlrp12 is protective against *Yersinia pestis* infection through Nlrp12’s activation of the inflammasome which results in secretion of IL-18 and IL-1β. But again, no protein-protein interactions of Nlrp12 within inflammasomes had been characterized (Vladimer et al., 2012). Thus, there is a further need to find and to characterize previously unreported Nlrp12-binding proteins. Knowing Nlrp12’s protein binding partners may help to gain mechanistic insight into pathways previously characterized as being regulated by Nlrp12. Moreover, identifying and validating potentially novel binding partners of Nlrp12 may suggest novel functions for Nlrp12.

In humans, Nlrp12 is mainly expressed in macrophages, DCs, and neutrophils. In mice, Nlrp12 is primarily expressed in DCs and neutrophils (Arthur et al., 2010; Hornick et al., 2018; Chen et al., 2019). The Nlrp12 protein has three domains, from N- to C-terminus: PYD, NBD, and LRR domains (Damiano et al., 2004; Janowski et al., 2013; Tuncer et al., 2014). It is generally accepted that the LRR domain, like Nlrp1’s LRR domain and Nlrp3’s LRR domain, is responsible for sensing ligands or other types of activators of Nlrps in modulating their role in an immunologic response (Martinon et al., 2004; Faustin et al., 2007; Kanneganti et al., 2007; Sharif et al., 2019). However, interestingly, it has been reported that without the LRR domain, Nlrp3 still can be activated by triggers of the canonical NF-κB pathway (Hafner-Bratković et al., 2018). The NBD domain is responsible for self-oligomerization when Nlrp is activated, and the
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PYD domain is responsible for regulating downstream signaling pathways (and thus, it is called the “interaction domain”) (Hafner-Bratkovič et al., 2018; Hornick et al., 2018). Here we used a truncated form of Nlrp12 (PYD + NBD) to identify possible protein-protein interactions that may regulate signaling pathways in which Nlrp12 is involved. In addition, to our knowledge, there are currently no protein-protein interaction studies that have been performed for this truncated form of Nlrp12 and may thus represent a new general approach to screening for protein-protein interactions involved in signaling complexes with Nlrps.

A yeast two-hybrid screen was performed to identify potential binding partners of Nlrp12’s PYD + NBD. Hck, a member of the Src family of non-receptor tyrosine kinases, was one of the top positive clones (“hits”) among all of the proteins that appeared to interact with Nlrp12. Further experiments were done to confirm the novel binding of Nlrp12 to Hck and to characterize key structural features of the binding interaction. In addition, co-expression of Hck with Nlrp12 resulted in a significant decrease of steady-state Nlrp12 protein expression levels. In consideration together with a bioinformatics analysis included here, a potential interaction between Nlrp12 and Hck may be involved in modulating the role of Hck in the pathogenesis of AML (Roversi et al., 2015; Lopez et al., 2016; Roversi et al., 2017).

Results

Nlrp12 interacts with Hck in the yeast two-hybrid assay

A human leukocyte cDNA library was used as prey in the screening of binding partners for Nlrp12 using the yeast two-hybrid assay. The basic principle of the yeast two-hybrid assay is that if the cDNA library contains clones that express proteins, or parts thereof, that interact with
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the co-expressed bait, the Nlrp12 PYD + NBD (Figure 1A) in this case, then the co-transformed yeast clones will grow. The cDNA clones from the library that supported growth would then be isolated and characterize, to identify what proteins interact with Nlrp12’s PYD + NBD. The leukocyte library was used because Nlrp12 is mainly expressed in leukocytes in humans (Tuncer et al., 2014; Linz et al., 2017; Hornick et al., 2018; Normand et al., 2018), and thus putative binding partners for Nlrp12 are also likely to be expressed there.

The screening resulted in 119 positive clones from the leukocyte cDNA library (“hits”) expressed from 48 distinct genes (Supporting information, Table S1) that were subsequently identified as encoding for proteins (or portions thereof) that potentially interact with Nlrp12’s PYD + NBD. The clones with the most hits are listed in Table 1. Among the 119 hits, which included repeated hits of the same genes, 9 were individual clones that encoded fragments of a member of the Src family of non-receptor tyrosine kinases, Hck, which was among the highest number of hits for a single gene (Figure 1B and Table 1). Moreover, of the 9 positive Hck clones, 5 were distinct Hck fragments (Figure 1C). Full-length Hck p61 was also obtained as a hit in another independent yeast two-hybrid screen (data not shown). Hck has two isoforms, p59 and P61, in Homo sapiens. P61 and p59 share the same transcript, but p61 uses an uncommon start codon CTG, and p59 uses the common one, ATG (Robbins et al., 1995), to give rise to two distinct transcripts. Endogenously expressed in phagocytes, Hck p59 is mainly localized to the plasma membrane, while Hck p61 is primarily localized to lysosomes, and both of them are also found in the Golgi apparatus (Carreno et al., 2000). It has been suggested that overexpression of p59 promotes the formation of membrane protrusions such as pseudopodia and overexpression of p61 induces podosome rosettes and triggers actin polymerization around lysosomes (Carreno et
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However, there are no differences between p61 and p59 in the region of Hck that appears to bind to Nlrp12’s PYD + NBD (see below), the C-terminal part of Hck. Thus, detecting a full-length clone of only p61 as a hit, opposed to p59, appeared to be coincidental.

In directed screens using the yeast two-hybrid assay, Nlrp12’s PYD + NBD domain binding to Hck C-terminal 40 amino acids appears to be selective for Nlrp12 and Hck binding to Nlrp12 appears to be selective for Hck

Initially, the Hck C-terminal 40 amino acids (“Hck C1 fragment”) was the shortest Hck fragment from a positive clone that interacted with the bait, Nlrp12’s PYD + NBD (Figure 2A). Nlrp3 and Nlrp8 were chosen based on the phylogenetic trees of the PYD + NBD domains of members of the Nlrp family: phylogenetically, Nlrp3’s PYD + NBD is the most closely related Nlrp PYD + NBD to that of Nlrp12, while Nlrp8’s PYD + NBD is among the furthest related PYD + NBD to that of Nlrp12 (Figure 2B). In Figure 2C, yeast clones grew on the 4 drop out (DO) (synthetic dropout (SD) plus amino acids supplements minus tryptophan, leucine, histidine, and adenine (-Trp-Leu-His-Ade) and 4DO + 3-Amino-1,2,4-triazole (3-AT) high stringency plates when the yeast were co-transformed with Nlrp12’s PYD + NBD and Hck C1 fragment; and thus, this result is indicative of an interaction between the two constructs. However, yeast clones were not observed on 4DO and 4DO + 3-AT plates when Nlrp3’s PYD + NBD and Hck C1 fragment were co-transformed, nor when Nlrp8’s PYD + NBD and Hck C1 fragment were co-transformed. These results suggest that the Hck C1 fragment binds to Nlrp12’s PYD + NBD, but not to those from either Nlrp3 or Nlrp8, results consistent with a selective interaction between Nlrp12’s PYD + NBD and the Hck C1 fragment.
Similarly, yeast clones again grew on the high stringency 4DO and 4DO + 3-AT plates when the Hck C1 fragment was co-transformed with Nlrp12’s PYD + NBD. However, clones did not grow on the 4DO and 4DO + 3-AT plates when the C1 fragment of other members of the Src family of non-receptor tyrosine kinases were co-transformed with Nlrp12’s PYD + NBD (Figure 2D). This experiment suggests that the interaction of Hck C1 fragment with Nlrp12 is selective for Hck; the C1 fragment of other members of the Src family of non-receptor tyrosine kinases did not bind to Nlrp12’s PYD + NBD (Figure 2D).

In directed screens using the yeast two-hybrid assay, amino acids F503, Q507, L510, and D511 of Hck are critical for the binding between Nlrp12’s PYD + NBD and Hck C-terminal 40 amino acids

The Hck C1 fragment were divided into four helical regions according to its atomic structure (Sicheri et al., 1997) (protein data bank (PDB) accession number: 1AD5): R1, R2, R3, and R4 (designated by us) (Figure 3A). As shown in the sequence alignments in Figure 3B, the primary amino acid sequences of R2, R3, and R4 are very well conserved among members of the Src family of non-receptor tyrosine kinases, while R1 is not. To characterize further the important structural features of Hck’s binding to Nlrp12’s PYD + NBD, all of the amino acids comprising each of the R2, R3, and R4 domains were mutated to alanine, but mutated one section at a time, and were denoted as the Hck M2 fragment, Hck M3 fragment, and Hck M4 fragment, respectively. The amino acids comprising the R1 region were mutated to those amino acids in the R1 region of the Src family non-receptor tyrosine kinase Lyn, which has the closest phylogenetic relationship to Hck (Figure 3C). All of the mutated constructs are illustrated in
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Figure 3D. Yeast still grew on the high stringency 4DO + 3AT plates when Nlrp12’s PYD + NBD was co-transformed with the Hck M1 fragment, Hck M2 fragment, and Hck M4 fragment. But, yeast could not grow when Nlrp12’s PYD + NBD was co-transformed with Hck M3 fragment (Figure 3E). These experiments suggested that the R1, R2, and R4 regions are dispensable for binding, while R3 may be important for binding. Further analysis was conducted to identify individual critical amino acids of Hck’s R3 region binding to Nlrp12’s PYD + NBD using alanine scanning mutagenesis. However, the intervening sequences between R1, R2, R3, and R4 were not examined for binding.

To identify the key amino acid residues involved in binding of R3 to Nlrp12’s PYD + NBD, each amino acid in R3 was individually mutated to alanine in an alanine scanning mutagenesis analysis, on the background of the Hck C1 fragment. As shown by the yeast two-hybrid assay, the mutations of F503A, Q507A, L510A, and D511A abolished the growth of yeast clones on the high stringency 4DO when these yeast strains were co-transformed with Nlrp12’s PYD + NBD (Figure 3F). These experiments suggest that these four amino acids are critical for binding. On the other hand, yeast clones still grew on the high stringency 4DO plates when the Hck C1 fragments with the Y505A, S508A, or V509A mutations were co-transformed with Nlrp12’s PYD + NBD (Figure 3F). These experiments suggest that Y505, S508, and V509 are dispensable for binding of Hck C1 fragment to Nlrp12’s PYD + NBD. Whether E504 and I506 are critical for binding is unfortunately not clear as the yeast did not grow on the control plates (SD/-Trp-Leu). Overall, however, these results suggest that the amino acids F503, Q507, L510, and D511 in Hck are critical for the binding of Hck C1 fragment to Nlrp12’s PYD + NBD.
On the other hand, these four amino acid residues are all conserved in all members of the Src family of non-receptor tyrosine kinases (Figure 3A), suggesting that, while they are important for binding, they cannot account for the specificity or selectivity of binding between Nlrp12 and Hck.

In addition, these four critical residues, as shown in Figure 3C, appear to be in the same plane along a helix, which may then form an interface that is important for binding to Nlrp12.

However, it is unclear whether the structure of the isolated Hck C-terminus used in the screens here will retain its structural features that it possesses in full-length Hck, due to the loss of intermolecular interactions for the C-terminal region. Moreover, for example, the amino acids R500, P501, T502, D512, T515, Q520, and Y521, are 100% conserved among all other members of the Src family of non-receptor tyrosine kinases and are therefore unlikely to play a role in specificity and selectivity of binding of Nlrp12 with Hck. But, it is possible that two or more amino acids in the intervening sections between the R1-R4 regions, which were not analyzed by mutagenesis, may then actually account for the specificity and selectivity of binding of Hck to Nlrp12. One example is that the combination of R496, P497, and Q522 is unique to Hck compared to other members of the Src family of non-receptor tyrosine kinases.

In directed screens using the yeast two-hybrid assay, the shortest fragment of Hck that binds to Nlrp12’s PYD + NBD is its C-terminal 30 amino acids

The shortest form of Hck that bound to Nlrp12’s PYD + NBD was determined by observing whether the yeast clones grew on high stringency 4DO or 4DO +3AT plates when Nlrp12’s PYD + NBD was co-transformed with Hck fragments C1 to C7 and N1 to N5 (Figure 4) (data of Nlrp12’s PYD + NBD co-transformed with Hck fragment C7 did not shown). Hck’s C1 to C7 fragments were gradually truncated from the N-terminus of the Hck C-terminal 40 amino acid...
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fragment (Figure 4A and 4B). Hck N1 to N5 fragments were gradually truncated from the C-terminus of the Hck C-terminal 40 amino acid fragment (Figure 4C and 4D). Yeast strains grew on high stringency 4DO or 4DO +3AT plates when either the Hck C1, C2, or C3 fragment was co-transformed with Nlrp12’s PYD + NBD. Thus, of all of the constructs screened, the shortest fragment of Hck that can apparently bind to Nlrp12’s PYD + NBD is the C-terminal 30 amino acids, as indicated by the Hck C3 fragment (Figure 4B and 4D). In addition, the C-terminal 5 amino acids also appear to be important for binding, as clones co-transformed with Nlrp12’s PYD + NBD with N1, with a deletion of the C-terminal 5 amino acids of Hck, did not grow on the high stringency 4DO and 4DO + 3AT plates (Figure 4D). The structures of both inactive Hck (PDB accession number: 1AD5) and active c-Src (PDB accession number: 1Y57), the prototypical member of this family of nonreceptor tyrosine kinases related to Hck, show that the C-terminal 4 amino acids are surface-exposed, indicating that these four amino acids that are critical for binding are available to interact with Nlrp12.

Nlrp12 co-immunoprecipitates with Hck from mammalian cells, and Nlrp12 is co-localized with Hck in mammalian cells

Nlrp12 and Hck could be co-immunoprecipitated when they were both heterologously co-expressed in 293T cells (Figure 5A). Also, semi-endogenous conditions were used for co-immunoprecipitations from cells in which Hck is endogenously expressed, and Nlrp12 was heterologously stably expressed in epitope-tagged form, such as in macrophage-like RAW 264.7 cells (Figure 5B), monocyte-like THP-1 cells (Figure 5C), and lymphocyte-like U937 cells (Figure 5D). Semi-endogenous conditions have been used in previous reports where Nlrp12 was shown to interact with TRAF3 (Allen et al., 2012), NIK (Lich et al., 2007), and IRAK-1(Ye et al.,...
In all cases, Nlrp12 co-immunoprecipitated with the p59 and p61 isoforms of Hck, but from these experiments, it is not clear that whether Nlrp12 interacts with p59, p61, or both isoforms of Hck. In U937 cells and THP-1 cells, since heterologous Nlrp12 expression level was low, adding phorbol 12-myristate 13-acetate (PMA) (1 µM for 24 hr) increased Nlrp12 expression level by stimulating the PMA-sensitive cytomegalovirus promoter of Nlrp12’s expression plasmid (Figure 5C and 5D). Taken together, these data suggest that Nlrp12 and Hck (either p59, p61, or both isoforms) can interact with each other in a mammalian cell system, a result that in part validates the findings from the yeast two-hybrid assay.

After heterologous co-expression of Nlrp12 with either the p61 or the p59 isoform of Hck in 293T cells, immunofluorescent staining of Nlrp12 and Hck showed that Nlrp12 can be co-localized with either p61 or p59 isoforms of Hck in most of the 293T cells (Figure 5E and Supporting Information Figure S1). The co-localization of Nlrp12 and Hck is consistent with an intracellular interaction between the two proteins. In addition, the lack of selectivity of Nlrp12 co-localizing with either p61 or p59 isoform of Hck is consistent with the results from the screening assay, where the putative binding region of Hck to Nlrp12 is identical in both the p61 and p59 isoforms.

The steady-state level of Nlrp12 protein is significantly decreased when Hck protein is co-expressed

Figure 6 shows that in 293T cells, the co-expression of Hck led to a significant decrease in steady-state protein expression levels of Nlrp12. Although, alternatively, the apparent decrease in expression level of Nlrp12 might be due actually to a decrease in the epitope-tag itself or a
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decrease in the expression of the epitope-tagged protein only. Interestingly, Hck protein levels
did not show an increasing level of expression upon transfection with increasing amounts of
plasmid DNA, possibly due to Hck’s maximum expression levels in these cells being achieved
with the lowest amounts of plasmid DNA, which may be resolved if the transfections are
performed with lower amounts of plasmid DNA.

In blood and marrow samples from patients with AML, Nlrp12 and Hck co-occur and are
co-expressed

The odds ratio test (a statistical test that defines the likelihood of association of two events)
determines whether two proteins either co-occur or are mutually exclusive in their expression.
The Fisher exact test is a statistical test that uses P-values to determine if two events, such as co-
ocurrence or mutual exclusivity, are quantitatively significantly associated. Applying these
tests to The Cancer Genome Atlas (TCGA) provisional data sets for AML patients (Cerami et al.,
2012a; 2013; Gao et al., 2013), Nlrp12 and Nlrp1 are the only two Nlrp family members that
show a statistically significant co-occurrence with Hck (Table 2). However, Nlrp1’s co-
ocurrence has a P value of 0.048, while Nlrp12’s co-occurrence has a P value of <0.001.
Therefore, Nlrp12 is most likely the only Nlrp family protein that shows co-occurrence with Hck
in samples from patients with AML.

Moreover, in this analysis, Nlrp12 is significantly co-expressed with Hck (Pearson coefficient =
0.80 and Spearman coefficient = 0.73, Pearson and Spearman coefficients show the possibility of
a linear relationship of two factors, Figure 7). This analysis is also consistent with Nlrp12 and
Hck co-occurring in the same patients with AML. However, Nlrp12 was not the only Nlrp
protein that shows significant co-expression with Hck. Nlrp1 and Nlrp3 also showed significant
coa-expression with Hck. This can be explained by the fact that co-expression is less stringent
than co-occurrence. In summary, Nlrp12 is the only Nlrp protein that shows co-occurrence with
Hck, and it also shows co-expression with Hck, although it is not the only member of the Nlrp
family to do so. Other data sets, including Hausera data, Metzeler 1 data, Metzeler2 data, and
Raponi data, were not available to evaluate the co-expression of Nlrp12 versus Hck, as these
other data sets did not have information regarding Nlrp12 expression.

Other potential binding partners of Nlrp12 from the yeast two-hybrid screen

Other potentially interesting and unique genes that whose products may interact with Nlrp12’s
PYD + NBD were identified in the yeast two-hybrid screen (Table 1 and Supporting information
Table S1). Table 1 only shows those hits with frequency larger than 2, and these hits were
analyzed through an Ingenuity Pathway Analysis (IPA) diagram (Figure 8). In the IPA diagram,
protein-protein interactions found by our yeast two-hybrid screen are connected by solid lines
and interactions between protein binding partners collated by IPA are connected by dashed lines.
In addition, those molecules that are associated with AML are colored purple. Interestingly,
Nlrp12 is also associated with AML from the database Catalogue of somatic mutations in cancer
(COSMIC). Although the mutation occurs in the NBD of Nlrp12, only 2 samples of these
mutations are observed within 175 samples.

The interaction of one of the putative binding partners to Nlrp12 identified in our screen, TRAF3
interacting protein 3 (TRAF3IP3), was validated in a co-immunoprecipitation experiment where
the FLAG-tagged form of TRAF3IP3 and V5-tagged form of Nlrp12 were overexpressed in
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293T cells. TRAF3IP3 was coimmunoprecipitated by adding mouse monoclonal anti-
TRAF3IP3 antibody, and a band that showed the same molecular weight size of Nlrp12 was
detected by rabbit monoclonal anti-V5 antibody (Figure 8). However, further validation of these
other putative interactors detected by the yeast two-hybrid needs to be done.

Discussion

In this paper, the yeast two-hybrid assay was used to screen for Nlrp12 binding partners. This
approach identified 48 genes as positive hits encoding proteins that could possibly bind to
Nlrp12. Among the hits, the Src non-receptor tyrosine kinase family member Hck was selected
to focus upon as a potentially interesting target, particularly given its association with
hematopoiesis and AML. Further direct screens found that Nlrp12 specifically binds to Hck, but
not to Nlrp8 or Nlrp3. Conversely, Hck, but not other members of the Src non-receptor tyrosine
kinase family, specifically binds to Nlrp12. Hck’s C-terminal 30 amino acids are sufficient for
binding to Nlrp12. And, amino acids F503, Q507, L510, and D511 of Hck are critical for
binding to Nlrp12. Co-immunoprecipitation experiments from mammalian cells confirmed that
Nlrp12 and Hck interact. Co-expression of Nlrp12 and Hck results in a significantly lower
steady-state expression level of Nlrp12; thus, the interaction may affect Nlrp12 protein stability.
Finally, a bioinformatics analysis shows that Nlrp12 and Hck essentially exclusively co-occur,
and mRNA levels for both are correlated in blood samples from AML patients. Together, these
data suggested that Nlrp12 and Hck protein and mRNA co-expression may have an effect on the
AML.
The binding data from yeast two-hybrid (Figure 3 and Figure 4) showed that the R2 and R3 regions are both important for binding, but we suspect that R3 region is the major region that is critical for binding, while R2 region may be an “auxiliary” region that is also critical for binding, because when we delete the R2 region but maintain the R3 region, the R3 region itself cannot maintain stable binding of Hck’s C-terminal 40 amino acids to Nlrp12’s PYD + NBD. Thus, binding of Nlrp12’s PYD + NBD to this fragment of Hck may occur initially through binding to R3, followed by stabilization of the interaction by secondarily binding to R2. This model is also consistent with the finding that when we mutated the R3 region, the binding of Hck’s C-terminal 40 amino acid fragment to the Nlrp12’s PYD + NBD was lost despite the R2 region being present. The above evidences are consistent with the model that R2 and R3 regions together are both necessary for binding, but the stabilization of binding to the R3 region requires the presence of R2.

The yeast two-hybrid screen with Nlrp12 also identified 47 other genes as potential interactors. According to the Search Tool for Recurring Instances of Neighbouring Genes (STRING) protein network and previous publications, Nlrp12 interactors include IRAK1 (Williams et al., 2005), NIK (Lich et al., 2007), TRAF3(Allen et al., 2012), heat shock protein 90 (HSP90) (Arthur et al., 2007), Fas associated factor 1 (FAF1) (Pinheiro et al., 2011), and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) proteins. IRAK1, NIK, TRAF3, and Heat shock protein (HSP) 90 were only found in experiments involving semi-endogenous protein co-immunoprecipitations with Nlrp12. FAF1 was found to be an interacting protein in NMR experiments by showing a difference in the chemical shift perturbation of FAF1’s ubiquitin-associated domains (UBA) domain alone and Nlrp12’s PYD domain bound to
Nlrp12 binds to Hck

FAF1’s UBA domain. And FAF1 was also found to interact with Nlrp12 in a yeast two-hybrid study where the PYD domain of different Nlrp proteins were screened (Kinoshita et al., 2006). However, in our study, IRAK1 (Shi et al., 2019), NIK (Fu et al., 2017), TRAF3 (Wang et al., 2018), HSP90 (Kinnaird et al., 2017), FAF1 (Ryu et al., 2003), and ASC (Martin et al., 2016) proteins were not found in our results of interacting proteins, although they are all expressed in leukocytes. Therefore, given these differences, the results from this yeast two-hybrid screen may provide a unique database for future investigations on the proteins that interact with Nlrp12, and the functional outcomes of these interactions.

How Hck co-expression affects Nlrp12 expression can be further investigated to determine the functional outcomes of the Nlrp12-Hck interaction relative to protein stability or degradation. For example, MG-132 and chloroquine, which are a proteasomal inhibitor and an inhibitor of lysosomal degradation, respectively, can be added to cells in these experiments to determine whether the decreasing protein expression level of Nlrp12 in the presence of Hck is due to proteasomal degradation or lysosomal degradation. In addition, an Nlrp12 stable cell line, with a typically lower level of Nlrp12 expression, compared to transiently transfected cells, can be used to test formally whether Nlrp12 protein expression levels are increasingly decreased when Hck is expression is increased. Moreover, the rate of Nlrp12 degradation, in the absence or presence of Hck, can be studied and compared in pulse-chase assays (Lich et al., 2007).

Given the interaction that was characterized between Nlrp12 and Hck, we sought to identify diseases in which their interaction may be relevant. AML is a type of blood and bone marrow cancer that is characterized by an unusually high level of circulating immature white blood cells.
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(Prada-Aristemdy et al., 2017). Hck is found to be highly expressed in hematopoietic stem cells of AML patients (Roversi et al., 2017). The overexpression of Hck is linked with the development of AML (Poh et al., 2015; Roversi et al., 2017). Therefore, Hck may be potentially used as a blood biomarker to identify those patients with potential to develop of AML disease.

In addition, activation of Hck and its signaling pathways often causes the development of AML (Dos Santos et al., 2008; Lopez et al., 2016). Hck has been reported to be involved in the feline McDonough sarcoma (fms)-like tyrosine kinase 3 (FLT3)-HCK- cyclin D-dependent kinase (CDK6) pathway. The mutation in FLT3-internal tandem duplications (FLT3-ITD) causes the activation of Hck, and subsequently results in overexpression of CDK6 in MV4-11 cells, a human AML cell line with the FLT3-ITD mutation. CDK6 siRNA resulted in MV4-11 cells with the FLT3-ITD mutation having a lower proliferation rate compared to MV4-11 control cells (Lopez et al., 2016). Also, it has been reported that in AML patients, Hck, Lyn, and Fgr phosphorylation levels are high (Dos Santos et al., 2008). In summary, the above evidence suggests that either high expression and/or high activation of Hck could occur in AML patients as well.

Our bioinformatics analysis revealed that Nlrp12 is likely the only member of the Nlrp family that shows significant co-occurrence with Hck at the level of mRNA expression. However, our cellular functional study revealed that Nlrp12 protein expression levels decreased when it is co-expressed with Hck. This discrepancy might be due Nlrp12 mRNA expression level not correlating with protein expression level (Greenbaum et al., 2003; Vogel and Marcotte, 2012; Liu et al., 2016). Nevertheless, these data are still consistent with the possibility that Nlrp12 may have an effect on AML through its binding to Hck. Nlrp12 and Hck co-expression and co-
Nlrp12 binds to Hck

collision may then also be relevant to the prognosis of AML. However, further analyses need to be performed to evaluate the ability of Nlrp12 and Hck to serve as biomarkers in the prognosis of AML.

Nlrp12 and Hck co-expression and co-occurrence may indicate that Nlrp12 and Hck mRNA or protein expression can impact the same pathway or both be regulated by the same molecule. For example, in the case two molecules exhibiting co-occurrence, they may interact in the same signaling pathway, for example, Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and glycogen synthase kinase 3 beta (GSK3β) showed co-occurrence in the AML provisional data, and they are in the same signaling pathway (Prochnicki and Latz, 2017). However, further analyses need to be performed to evaluate the ability of Nlrp12 and Hck to serve as biomarkers in the prognosis of AML.

Although many Hck inhibitors have been developed, they often lack specificity, with Hck inhibitors also inhibiting other members of the Src non-receptor tyrosine kinase family (Hu et al., 2004; Pene-Dumitrescu et al., 2008; Saito et al., 2013; Patel et al., 2017; Roversi et al., 2017; Naganna et al., 2019). If the potential for the development of AML can be decreased when Nlrp12 does not bind to Hck, then an inhibitor to the interaction between Nlrp12 and Hck can be designed. On the other hand, if the potential for AML is increased when Nlrp12 and Hck does not bind, then a drug that facilitates Nlrp12 and Hck interaction could be developed. By this approach, the non-specificity problem with Nlrp inhibitors may still exist but perhaps to a smaller extent, as Nlrp proteins are less conserved overall compared to the members of the Src non-receptor tyrosine kinase family. For example, the amino acid identity between Nlrp12’s
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PYD + NBD versus another Nlrp PYD + NBD most closely related to Nlrp12, Nlrp3’s PYD + NBD, is 54%. But, the protein identity between the Hck C1 fragment versus the one with the closest relationship, the Lyn C1 fragment, is 78%.

In conclusion, this paper reports the preliminary findings of Nlrp12 interacting with Hck, and Nlrp12 co-occurs with Hck in AML patients. The binding data presented here will expand the database regarding the interactors of Nlrp12, and additional bioinformatic and functional in vitro and in vivo analyses may shed light on the functional consequence of the interaction of Nlrp12 with Hck.

Material and Methods

Reagents and cells

U937, THP-1, RAW 264.7, and 293T cells were all purchased from American Type Culture Collection (ATCC) (Manassas, VA). pCDNA-Nlrp12 plasmid and pCDNA-Nlrp3 plasmid were gifts from Dr. Jenny P. Y. Ting (University of North Carolina-Chapel Hill). pCDNA-Nlrp8 plasmid was purchased from Genescript (Piscataway, NJ). All other chemicals were reagent grade. The mouse monoclonal anti-FLAG antibody and the rabbit monoclonal anti-FLAG antibody were purchased from Sigma-Aldrich (St. Louis, MO). The mouse monoclonal anti-Hck antibody, the mouse monoclonal anti-TRAF3IP3 antibody, and the mouse IgGκ binding protein-horseradish peroxidase (HRP) for detecting the anti-Hck antibody were purchased from Santa Cruz Biotechnology (Dallas, TX). The rabbit monoclonal anti-V5 antibody and polyclonal rabbit HRP-conjugated anti-mouse IgG secondary antibodies were purchased from Cell Signaling (Beverly, MA). The goat anti-rabbit IgG HRP-linked secondary antibody was
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purchased from Cell Signaling. The Clean-Blot™ IP Detection Reagent was purchased from Thermo Fisher Scientific (Grant Island, NY). Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 488 and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555 were purchased from Thermo Fisher Scientific (Grant Island, NY). The 4’6-diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from Thermo Fisher Scientific.

Cloning

Nlrp12’s PYD + NBD (a.a. 1-531) was amplified by polymerase chain reaction (PCR) (primers see Table 3), from a pCDNA-Nlrp12 plasmid, was digested with enzymes NdeI and SalI, and subcloned into the pGBKT7 vector (Takara). The sequence encoding the PYD + NBD of Nlrp3 (a.a. 1-536) was amplified by PCR (see table 3 for primers), digested with enzymes NdeI and BamHI, and subcloned in the pGBKT7 vector. The sequence encoding the PYD + NBD of Nlrp8 (a.a. 1-527) was amplified by PCR (see Table 3 for primers), digested with enzymes EcoRI and BamHI, and subcloned into the pGBKT7 vector. The entire sequences of the C-terminal 42 amino acids of Fgr, Src, Yes, and Fyn, and 40 amino acids of Blk, Lck, Lyn, and Hck (The C terminal 40 (or 42) amino acids is called “C1”), and Hck constructs C2, C3, C4, C5, C6, C7, N1, N2, N3, N4, N5, M1, M2, M3, M4, and Hck C1 mutations for alanine scanning with F503A, E504A, Y505A, I506A, Q507A, S508A, V509A, L510A, and D511A, were all synthesized from IDT DNA (Coralville, IA) and cloned into the pACT2 vector (Takara). Nlrp12 was amplified by PCR (see Table 3 for primers), digested with AflII and XbaI, and subcloned into pEFires-P-3FLAG (Hobbs et al., 1998) and pEFires -P-V5 (Hobbs et al., 1998). 3FLAG and V5 are homemade, i.e., added later into pEFires-P. The peptide sequence of the 3FLAG
epitope is DYKDDDKDYKDDDDKDYKDDDDK. The peptide sequence of the V5 epitope is GKIPNPLLGLDST. The multiple cloning sites of pEFiRES-P were modified according to our needs. Sequences encoding Hck p61 and p59 isoforms were amplified by PCR (see Table 3 for primers), digested with AfIII and XbaI, and subcloned into pEFiRES-3FLAG vector. Final plasmids were sequenced to confirm the constructs.

Yeast two-hybrid assay

Yeast transformed with the plasmid PGBKT7- Nlrp12’s PYD + NBD

Yeast strain AH109 (Takara, Mountain View, CA) was stored frozen at -80°C. Upon use, it was streaked onto an agar plate supplemented with yeast extract, peptone, dextrose, and adenine hemisulfate (YPDA) (Takara), which was then placed in a 30°C incubator overnight. The growing colonies were picked and placed into 10 ml of YPDA media, incubated overnight with shaking at 30°C. Then they were transformed with Nlrp12’s PYD + NBD plasmid, following recommendations in the Takara manual (Matchmaker™ Gal4 Two-hybrid System 3 and Libraries User Manual). A 4ml of 1 M of 3-AT was added into 800 mL of the YPDA medium that were used for making the plates due to a leakage problem. 3-AT is a competitive inhibitor for the product of the HIS3 reporter gene. Yeast cells were lysed using a trichloroacetic acid (TCA) method, and western blots were performed to check Nlrp12’s PYD + NBD protein expression (data not shown).

Library scale transformation of cDNA library into yeast that have been transformed with the PGBKT7- Nlrp12 PYD + NBD plasmid
A human leukocyte Matchmaker™ cDNA library was purchased from Takara. The library was titered according to the Matchmaker™ Gal4 Two-hybrid System 3 and Libraries User Manual.

The colony-forming units (CFU) for the library were calculated to be about $3.1 \times 10^9$ CFU/ml.

Four large scale transformations (equal to one library scale) were done at the same time following the Matchmaker™ Gal4 Two-hybrid System 3 and Libraries User’s Manual. To calculate the efficiency of yeast two-hybrid (i.e., the number of colons obtained per µg of library plasmid DNA) and to check the expressions of “bait” and “prey” plasmids, small amounts of the solution, diluted 10X, 100X, 1000X, and 10,000X were plated in SD/-Trp-Leu 2DO (drop out) plates. The yeast two-hybrid efficiency obtained was $3 \times 10^6$ CFU per µg of library plasmid DNA. In addition, if the yeast grew on the SD/-Trp-Leu-His-Ade + 3-AT plates, the “bait” and “prey” plasmids were considered to be interacting with each other.

Small scale transformation

The yeast two-hybrid co-transformation on a small scale for all of the direct screenings was performed following the small-scale transformation protocol in the Matchmaker™ Gal4 Two-hybrid System 3 & Libraries User’s Manual. The direct screens were to test whether Nlrp12 PYD + NBD and Hck specifically interact; identification of Hck F503, Q507, L510, and D511 being critical for binding with Nlrp12 PYD + NBD; and the characterization of the Hck C-terminal 30 amino acid fragment binding to Nlrp12 PYD + NBD.

Cell transfection

293T cells, THP-1 cells, U937 cells, and RAW 264.7 cells were seeded at a density of $5 \times 10^5$ per well in a 6-well plate. Transfection followed the Lipofectamine® 3000 reagent (Thermo
Fisher Scientific) protocol with the following modifications. Briefly, 3.75 µl of Lipofectamine®
3000 was added into 125 µl of Opti-MEM® medium (Thermo Fisher Scientific). 2.5 µg of
plasmid DNA (prepared by isolation through cesium chloride (CsCl) density gradient
purification (Brakke, 1951) and 5 µl of P3000™ reagent were added into another aliquot of 125
µl of Opti-MEM® medium. Then these two tubes were mixed together, and the mixed tube was
incubated at room temperature for 15 min. 250 µl of this mixture was added to the cells. The
cells were incubated at 37 °C with 5% CO₂ for two days until analysis.

Nlrp12 stable cell lines
THP-1 cells, U937 cells, and RAW 264.7 cells were transfected with pEFIRES-P-Nlrp12-
3FLAG plasmid, and 2.5 µg/ml puromycin (Invitrogen™ by Thermo Fisher Scientific) was added.
After two weeks, the cells were considered to be stably expressing Nlrp12 after screening for
expression. The stable cells were “pooled” clones, and they are maintained by adding 2.5 µg/ml
puromycin.

Cell lysis and immunoprecipitation
After trypsinization, cells were lysed in 500 µl of lysis buffer (1% NP-40, 4mM Tris-HCl, pH 8.0,
150 mM NaCl). Cells were then treated with freeze-thaw cycles, i.e., frozen in the -80°C freezer
for 5 min and thawed at 37°C in a water bath for 3 min, for a total of five times. Cells were then
centrifuged at 12,000 x g for 10 min at 4°C. After centrifugation, the supernatant was saved at -
20°C until further use.
For immunoprecipitations, cell lysates were pre-cleared by adding 40 μl of protein A/G agarose beads (Thermo Fisher Scientific) from a 50% slurry, and the sample was rotated for 1 hr at 4°C. The sample was spun at 1000 x g for 1 min. Then, 30 μl of pre-cleared cell lysate were removed and run on immunoblotting as a control. The remaining cell lysate was incubated with 1 μg anti-Hck mouse monoclonal primary antibody overnight. On the second day, 30 μl of protein A/G agarose, from a 50% slurry and previously washed with lysis buffer three times, were added to the tube, and the tube was incubated for 3 hrs at 4°C. The beads were then washed with lysis buffer, but with the NaCl concentration increased to 500 mM. The tube was briefly centrifuged at 1000 x g for 1 min., and the supernatant was removed. This step was performed for a total of 5 times. The proteins were eluted from the beads by addition of SDS-PAGE sample buffer.

**Immunoblotting**

Protein concentrations were determined by the Pierce™ BCA protein assay (Thermo Fisher Scientific). About 20 μg of cell lysate samples and immunoprecipitated samples were loaded onto the 8% acrylamide gel. After overnight transfer of the proteins from an SDS-gel to a 0.45 μm pore size polyvinylidene difluoride (PVDF) membrane, the membrane was blocked in for 1 hr at room temperature 5% non-fat dry milk dissolved in (Tris-buffered saline with Tween 20, pH=8.0) (TBST) (Sigma-Aldrich). Mouse monoclonal anti-FLAG antibody (diluted 1:1000) and mouse monoclonal anti-Hck antibody (diluted 1:1000) were added and the membrane incubated overnight. For detection of bands in the cell lysate, rabbit anti-mouse IgG HRP-linked secondary antibody (diluted 1:2000) (for anti-FLAG antibody) and mouse IgGκ binding protein-HRP secondary antibody (for anti-Hck antibody) (diluted 1:1000) were added, and the membrane was incubated for 1 hr at room temperature. For immunoprecipitations, Clean-Blot™ IP detection
reagent (HRP) secondary antibody (1:300) (Thermo Fisher Scientific) was used. The detection signal was visualized by using enhanced chemiluminescence (ECL) reagent (Thomas Scientific, Swedesboro, NJ). ImmobLOTS were analyzed by chemiluminescence on a Bio-Rad ChemiDoc™ Touch Gel imaging system (Bio-Rad, Hercules, CA).

**Immunofluorescence**

A protocol for immunofluorescence was followed ([https://www.cellsignal.com/contents/resources-protocols/immunofluorescence-general-protocol/if](https://www.cellsignal.com/contents/resources-protocols/immunofluorescence-general-protocol/if)), with the following modifications. Coverslips were autoclaved and coated with 0.1 mg/ml of poly-D-lysine hydrobromide (mol wt 70,000-150,000) (Sigma-Aldrich). Cells were plated onto the coverslips. Fixation was stopped by adding 50 mM NH₄Cl in PBS for 15 min at room temperature. Fixed cells were permeabilized by adding 0.5% Triton®-X-100 in PBS for 10 min. The cells were then washed with PBS three times and blocked with 0.1% of BSA in PBS at room temperature for 1 hr. The rabbit monoclonal anti-FLAG antibody (diluted 1:100) and mouse monoclonal anti-Hck antibody (diluted 1:100) were added to each cover slip and incubated overnight. Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 488 (diluted 1:100) and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555 (1: 100) were added. After incubation with secondary antibodies, the cells were washed five times with PBS. During the first step, DAPI (Invitrogen™ by Thermo Fisher Scientific) was also added at 1:1000 in PBS for 2 min. Finally, the coverslips were mounted in ProLong™ Gold Antifade mounting medium (Life Technologies Corporation by Thermo Fisher Scientific, Eugene, OR). The stained slides were viewed on a
ZEISS LSM 880 (Pleasanton, CA) with Airyscan. The images were viewed and processed using Fiji software (Schindelin et al., 2012).

IPA diagram

Yeast two-hybrid data were analyzed by IPA software (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

Statistical analysis

The Spearman coefficient and Pearson coefficient used for co-expression was generated by cBioPortal website (Cerami et al., 2012b; 2013; Gao et al., 2013). Clinical samples from the Oncomine website (https://www.oncomine.org) were downloaded and checked for Nlrp12 expression levels and Hck levels. The Fisher exact test and the odds ratio test used for co-occurrence were generated by the cBioPortal website. Analysis of variance (ANOVA) was performed to check whether a significant difference of the treatment exists among all the groups. A p-value < 0.05 was considered statistically significant. If P < 0.05, then the Tukey test was used to determine whether a pair-wise difference exists. Quantification of the western blot bands was performed by FIJI software (Schindelin et al., 2012) and made into graphs by Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Acknowledgement
Thank you to Dr. Houda Alachkar for providing valuable suggestions to this paper. We also thank Ms. Meng Li and Dr. Yibu Chen at the University of Southern California’s Norris Medical Library for their suggestions for bioinformatic analysis. Appreciation is given to Mr. Noam Morningstar-Kywi and Dr. Ian Haworth for their helps on structural analysis.

Abbreviations

- 3-AT, 3-Amino-1,2,4-triazole
- AML, acute myeloid leukemia
- ANOVA, analysis of variance
- ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
- ATCC, American Type Culture Collection
- C1, self-named truncations of C-terminal 40 (or 41) amino acids fragment of Src family non-receptor tyrosine kinases
- C2-C7 and N1-N5, self-named truncations Hck C1 fragment
- CD40, cluster of differentiation 40
- CDK6, cyclin D-dependent kinase
- CFU, colony-forming units
- COSMIC, catalogue of somatic mutations in cancer
- CsCl, cesium chloride
- DAMP, damage-associated molecular patterns
- DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride
- DC, dendritic cells
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634 DO, drop out (2DO: SD/-Trp-Leu; 4DO: SD/-Trp-Leu-His-Ade);
635 ECL, enhanced chemiluminescence
636 FAf1, Fas associated factor 1
637 FLT3-ITD, FLT3-internal tandem duplications
638 FLT3, feline McDonough sarcoma (fms)-like tyrosine kinase 3
639 GSK3b, glycogen synthase kinase 3 bet
640 Hck C1 fragment, Hck C-terminal 40 amino acids
641 Hck, Hematopoiesis cell kinase
642 HRP, horseradish peroxidase
643 HSP90, heat shock protein 90
644 IL, interleukin
645 IPA, ingenuity pathway analysis
646 IRAK1, IL-1 receptor-associated kinase 1
647 KO, knock out
648 KRAS, Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
649 LRR, leucine rich repeat domain
650 M1, the amino acids in the R1 region were mutated to the amino acids identical to the R1 region in Lyn;
651 M2, the amino acids in the R2 region were all mutated to Alanine
652 M3, the amino acids in the R3 region were all mutated to Alanine
653 M4, the amino acids in the R4 region were all mutated to Alanine
654 NBD, nucleotide binding domain
655 NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
Nlrp12 binds to Hck

NIK, NF-κB-inducing kinase

NLR, nucleotide binding domain and leucine-rich repeat

Nlrlp, NLR pyrin domain-containing protein

PAMP, pathogen-associated molecular patterns

PCR, polymerase chain reaction

PDB, protein data bank

PMA, phorbol 12-myristate 13-acetate

PRR, pattern recognition receptors

PVDF, polyvinylidene difluoride

PYD, pyrin domain

R1, R2, R3, and R4, self-named four regions in the Hck C terminal 40 amino acids fragment

SD, synthetic dropout

SD/-Trp-Leu, SD base plus amino acids supplements minus tryptophan and leucine

SD/-Trp-Leu-His-Ade, SD base plus amino acids supplements minus tryptophan, leucine, and histidine, and adenine

STRING: search tool for recurring instances of neighboring genes

TBST: tris-buffered saline with Tween 20, pH=8.0

TCA, trichloroacetic acid

TCGA, the cancer genome atlas

TLR, toll-like receptor

TRAF3, tumor necrosis factor receptor associated factor 3

TRAF3IP3, TRAF3 interacting protein 3

UBA, ubiquitin-associated domains
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WT, wild type

YPDA, yeast extract, peptone, dextrose, and adenine hemisulfate.

Conflict of Interest

The authors declare no conflict of interest regarding publication of this article.

Author Contributions Statement

YZ design the experiment and wrote and revised the manuscript. CO helped to design the experiments and to revise the manuscript.

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848 34
Table 1: yeast two hybrid results (hit frequency >= 3)

<table>
<thead>
<tr>
<th>Name</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Deleted in azoospermia (DAZ) associated protein 2 (DAZAP2)</td>
<td>10</td>
</tr>
<tr>
<td>Filamin A, alpha (FLNA)</td>
<td>9</td>
</tr>
<tr>
<td>Hematopoietic cell kinase (HCK) proto-oncogene, Src family tyrosine kinase (HCK)</td>
<td>9</td>
</tr>
<tr>
<td>Chaperone DnaJ (a.k.a., heat shock protein 40 kD(Hsp40)) homolog, subfamily B,</td>
<td>9</td>
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Nlrp12 binds to Hck

<table>
<thead>
<tr>
<th>Nlrp family proteins</th>
<th>Co-occurrence (C) or mutual exclusivity (M)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nlrp1</td>
<td>C</td>
<td>0.048</td>
</tr>
<tr>
<td>Nlrp2</td>
<td>M</td>
<td>0.546</td>
</tr>
<tr>
<td>Nlrp3</td>
<td>C</td>
<td>0.415</td>
</tr>
<tr>
<td>Nlrp4</td>
<td>M</td>
<td>0.936</td>
</tr>
<tr>
<td>Nlrp5</td>
<td>M</td>
<td>0.877</td>
</tr>
<tr>
<td>Nlrp6</td>
<td>M</td>
<td>0.670</td>
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</tbody>
</table>

Table 2: Co-occurrence of mRNA expression level of Hck and all of the Nlrp family proteins (from the provisional data, assessed on July 11 2019)
Nlrp12 binds to Hck

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nlrp12 5’ primer (cloned into PGBKT7)</td>
<td>GATCTACATATGCTACGAACCGCAGGCAG</td>
</tr>
<tr>
<td>Nlrp12 3’ primer (cloned into PGBKT7)</td>
<td>GATGTAGTCGACTCACCCCTCGTCCAGGATATAGTACATAGCT</td>
</tr>
<tr>
<td>Nlrp3 5’ primer (cloned into pGBKT7)</td>
<td>GATCTACATATGAAGATGGCAAGCACCACGCTGCA</td>
</tr>
<tr>
<td>Nlrp3 3’ primer (cloned into PGBKT7)</td>
<td>GATGTAGATCCCTCACAGCAGGTAGTACATGGCGGCAAAGA</td>
</tr>
<tr>
<td>Nlrp8 5’ primer (cloned )</td>
<td>GATCTAGAATTCATGAGTGACGTGAATCCACCCTCTG</td>
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### Table

<table>
<thead>
<tr>
<th>Nlrp12 binds to Hck</th>
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<tr>
<td>Nlrp12 3’ (cloned into PGBKT7)</td>
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<td>GATGTA<strong>GCTC</strong>TCAGAGTCTTGTGGGAAACAGAGAACA</td>
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<tr>
<td>Nlrp12 5’ (cloned into pEFIES-P-3FLAG and pIRES-V5)</td>
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<td>GTCCAGCTTAAGATGCTACGAAACCAGCGGAG</td>
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<td>Nlrp12 3’ (cloned into PIRES-3FLAG and pEFIES-P-V5)</td>
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<tr>
<td>CTGAATTCTAGAGCAGCCAATGTCCAAATAAGG</td>
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<td>Hck p61 5’ (cloned into pEFIES-P-3FLAG)</td>
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<tr>
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#### Figure legends:

**Figure 1:** (A) Schematic drawing of the domain structure of full-length Nlrp12 (top) and the domains used as the construct for the yeast two-hybrid screen (bottom). PYD: Pyrin domain; NBD: Nucleotide binding domain; and LRR: Leucine rich repeat domain. (B) The domain structures of the two alternatively spliced isoforms of Hck, Hck p59 and Hck p61. (C) Identification of the clones of Hck interacting with Nlrp12 (“hits”) obtained from the yeast two-hybrid screen. Among the nine of the clones positive for Hck, one clone encoded for the smallest interacting fragment of
Hck, the C-terminal 40 amino acids of Hck. The numbers in parentheses indicate the total number of hits obtained in the screen.

Figure 2: Specificity of binding of Nlrp12 with Hck. (A) Schematic drawing of Hck (p61 isoform, top) and, for comparison, the region of Hck (“Hck C1 fragment,” lower) used for testing the specificity of binding of Hck to other members of the Nlrp family of proteins. The numbers represent the amino acid residues of the protein or protein fragment (B) Phylogenetic tree showing the relationships among the PYD + NBD region of all of the members of the human Nlrp family of proteins. The phylogenetic tree was produced through Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). For each Nlrp family member, the PYD + NBD of the longest isoform was selected for generating the phylogenetic tree. Nlrp3 is phylogenetically the closest Nlrp to Nlrp12, while Nlrp8 is phylogenetically among the most distant from Nlrp12. Nlrp3 and Nlrp8 were highlighted in red color. (C) When Nlrp12’s PYD + NBD and Hck C1 fragment were co-transformed into yeast, they grew on the highest stringency plates (4DO + 3AT), consistent with an interaction between the two constructs. On the other hand, neither Nlrp3’s PYD + NBD co-transformed with the C1 fragment nor Nlrp8’s PYD + NBD co-transformed the C1 fragment grew on the highest stringency plates. These results are consistent with a specific interaction between Nlrp12 and the Hck C1 fragment, but not with the other Nlrps. (D) Yeast clones grew on the high stringency plates 4DO + 3AT when Hck’s C1 fragment and Nlrp12’s PYD + NBD were co-transformed, but they did not grow on the high stringency 4DO + 3AT plates when the C1 fragments of other members of the Src family of non-receptor tyrosine kinases were co-transformed with Nlrp12’s PYD + NBD. These results are again consistent with a specific interaction between Nlrp12 and Hck.
**Figure 3:** (A) Structure of the Hck C1 fragment generated by Chimera from the University of California, San Francisco (version 1.13.1), extracted from its structure within the entire Hck protein. Hck C1 fragment was thus divided into four helical domains: R1, R2, R3, and R4. The side chains of amino acids F503, Q507, L510, and D511 are shown. However, it is not clear whether the C1 fragment retains the structural features shown here when it expressed on its own.

(B) Primary amino acid sequence alignments showing of the C1 fragments of all members of the Src non-receptor tyrosine kinase family (40 amino acids for Hck, Blk, Lck, and Lyn; 42 amino acids for Fgr, Src, Yes, and Fyn). The black lines indicate the R1, R2, R3, or R4 regions. The arrows denote the four critical amino acids for binding of Hck C1 fragment to Nlrp12’s PYD + NBD. (C) Phylogenetic tree showing the relationship among all of the C1 fragments of the members of the Src family of non-receptor tyrosine kinases. The phylogenetic tree was produced through Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Hck was highlighted in red color. (D) Four sets of distinct mutations were made within the Hck C1 fragment. M1: the amino acids in the R1 region were mutated to the amino acids identical to the R1 region in Lyn (highlighted in red color); M2: the amino acids in the R2 region were all mutated to alanine (Ala); M3: the amino acids in the R3 region were all mutated to Ala; and M4: the amino acids in the R4 region were all mutated to Ala. (E) Yeast did not grow on high stringency 4DO + 3AT plates when Hck C1 fragment with the M3 mutations was co-transformed with Nlrp12’s PYD + NBD. But yeast can grow on the high stringency 4DO + 3AT plates when Hck C1 fragment contained either M1, M2, or M4 mutations, suggesting that the R3 region within the C1 fragment was necessary for binding to Nlrp12 NBD + PYD. (E) Yeast did not grow on high stringency 4DO plates when Nlrp12’s PYD + NBD was co-transformed with Hck
C1 fragment with single Ala substitutions at either F503, Q507, L510, or D511. But yeast grew on high stringency 4DO plates when Nlrp12’s PYD + NBD was co-transformed with Hck’s C1 fragment in which the following amino acids in the R3 region were individually mutated to Ala: Y505, S508, or V509. Yeast did not grow on either 2DO nor 4DO plates when Nlrp12’s PYD + NBD was co-transformed with Hck C1 fragment in which either E504 or I506 was individually mutated to Ala, making the results with these particular mutants inconclusive.

Figure 4: (A) Schematic drawing of Hck truncations comprising the C1 to C7 fragments. (B) Yeast grew on the high stringency 4DO plates and 4DO +3AT plates when Hck’s C-terminal 40 amino acid fragment (C1), Hck’s C-terminal 35 amino acid fragment (C2), and Hck’s C-terminal 30 amino acid fragment (C3), individually, were co-transformed with Nlrp12’s PYD + NBD. But yeast did not grow on the high stringency 4DO plates nor 4DO+ 3AT plates when C4 to C7 fragments were co-transformed to with Nlrp12’s PYD + NBD. The lack of yeast growth upon co-transformation of the C7 fragment and Nlrp12 are not shown. (C) Schematic drawing of Hck truncations comprising the N1 to N5 fragments. (D) Yeast grew on the high stringency 4DO plates and 4DO +3AT plates when Hck’s C-terminal 40 amino acid fragment (C1) was co-transformed with Nlrp12’s PYD + NBD (control). But yeast did not grow on the high stringency 4DO plates nor 4DO+ 3AT plates when N1 and N5 fragments were co-transformed to with Nlrp12’s PYD + NBD, suggesting that the very C-terminal 5 amino acids are also necessary for binding of Nlrp12. The colored boxes represent different fragments that were deleted each time.

Figure 5: Nlrp12 co-immunoprecipitates with Hck and co-localizes with Hck by immunofluorescence. (A) Nlrp12 co-immunoprecipitated with Hck when both proteins were
Nlrp12 binds to Hck

transiently co-expressed in 293T cells, with Nlrp12 having to be expressed as epitope-tagged forms. Cells were lysed using a non-denaturing cell lysis buffer (see Material and Methods). Co-immunoprecipitations were done using a mouse monoclonal anti-Hck antibody followed by protein A/G agarose. The immunoprecipitated proteins were analyzed by immunoblotting. The same method was used for experiments shown in (B), (C), and (D). (B) Nlrp12 co-immunoprecipitated with endogenous Hck in macrophage-like RAW 264.7 cells, when Nlrp12 is exogenously and stably expressed. Arrows show the two isoforms of Hck p59 and p61 that were immunoprecipitated by the anti-Hck antibody, although it is not clear whether Nlrp12 co-immunoprecipitated with one form or the other, or both. (C) Exogenously and stably expressed Nlrp12 co-immunoprecipitated with endogenously expressed Hck in monocyte-like THP-1 cells. In these cells, phorbol 12-myristate 13-acetate (PMA) (1µM) was added to enhance the expression of Nlrp12 under the PMA-sensitive cytomegalovirus promoter of the Nlrp12 plasmid. (D) Left panel shows that Nlrp12 was exogenously and stably expressed in lymphoblast-like U937 cells. Right panel shows that Nlrp12 co-immunoprecipitated with endogenously expressed Hck in U937 cells after PMA was added to 1µM, as was done for THP-1 cells, to enhance expression of Nlrp12. (E) Immunofluorescent images showing the colocalization of a cohort of Nlrp12 with either Hck p59 or p61. Hck was immunolabeled with a mouse monoclonal anti-Hck antibody, followed by a secondary goat anti-mouse IgG (H+L) cross-adsorbed, conjugated to Alexa Fluor 488. For Nlrp12, the primary antibody was a rabbit monoclonal anti-FLAG antibody, and the secondary antibody was a goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 555. Nuclei were counterstained by DAPI.
Figure 6: The co-expression of Hck protein significantly decreased the steady-state protein expression levels of Nlrp12. (A) Epitope-tagged Nlrp12-V5 and Hck p61 were co-transfected into 293T cells. Cell lysates were immunoblotted with rabbit monoclonal anti-V5 antibody for Nlrp12 and mouse monoclonal anti-Hck antibody. Comparing lane 2 (basal level of Nlrp12 expression) with lanes 5, 6, and 7, co-expression of Hck protein leads to a significant decrease of protein expression levels of Nlrp12 over three time are they co-expressed. Increasing protein expression of Hck, relative to the plasmid DNA added (ramp: lanes 5, 6, and 7; 2.5-20 μg of DNA) was not observed possibly because the amount of plasmid for Hck transfection were already too high, resulting in the maximum expression of Hck’s at all plasmid DNA amounts. The figure is a representative of one of three results. (B) Quantification of the Nlrp12 protein bands (mean + standard error) to show that Nlrp12 protein expression levels decreased in the presence of co-expressed Hck, relative to equal amounts of cell lysate being assayed. The Nlrp12 protein expression levels are indicated as arbitrary units. (C) Quantification of the Hck protein bands (mean + standard error) confirming that Hck protein expression levels did not change although the amount of plasmid DNA for Hck increased. Figure (B) and (C) were generated by Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Twenty μg of cell lysate proteins were loaded for each lane. ANOVA and Tukey tests were used to compare the Nlrp12 and Hck protein bands.

Figure 7: Nlrp12 is co-expressed with Hck in acute myeloid leukemia (AML) patient samples. Both Nlrp12 expression and Hck expression data are shown as log2 values. The data are RNA seq data obtained from cBioportal (provisional) database assessed on July 11, 2019. The graph was taken from the cBioportal website. Spearman coefficient, indicating the association of
Nlrp12 binds to Hck

Nlrp12 and Hck mRNA expression level (if Nlrp12 and Hck mRNA expression levels are non-parametric) = 0.80. Pearson coefficient, indicating the degree of the linear relationship between the Nlrp12 mRNA expression level and Hck mRNA expression level (if Nlrp12 and Hck mRNA expression levels are parametric) = 0.73. The p-values for Spearman coefficient and Pearson coefficient are both < 0.001.

Figure 8: (A) Ingenuity Pathway Analysis (IPA) displaying the 13 distinctive hits from the total results of the yeast two-hybrid screen for proteins that interacted with Nlrp12. The figure only shows the Nlrp12-interacting proteins with hits that have a frequency larger than 2. The solid lines indicate interacting proteins found through our yeast two-hybrid screen. The dashed lines are the Nlrp12-interacting proteins previously reported in published data. The purple-colored molecules are those associated with AML. (B) To validate an interaction shown in the IPA diagram, tumor necrosis factor receptor-associated factor 3 interacting protein 3 (TRAF3IP3)-3FLAG and Nlrp12-V5 were co-immunoprecipitated from co-transfected 293T cells. A non-denaturing cell lysate buffer (see Material and Methods) was used to lyse the cell, and co-immunoprecipitations were performed with a mouse monoclonal anti-TRAF3IP3 antibody followed by protein A/G agarose. The immunoprecipitates were analyzed for Nlrp12 by immunoblotting with rabbit monoclonal anti-V5 antibody. The results from the co-immunoprecipitation are consistent with a confirmation of an interaction between TRAF3IP3 and Nlrp12.
Figure 1

(A) Full-length Nlrp12

(B) Nlrp12 bait construct

(C) a.a. 417-525 (1)
    a.a. 450-525 (1)
    a.a. 449-525 (1)
    a.a. 475-525 (2)
    a.a. 486-525 (4)

Figure 1
Figure 2

(A) P61 Hck

(B) Nlfp11
Nlfp5
Nlfp7
Nlfp2
Nlfp1
Nlfp6
Nlfp10
Nlfp12
Nlfp3
Nlfp13
Nlfp8
Nlfp14
Nlfp9
Nlfp4

(C) Vector
Nlfp12’s PYD + NBD
Nlfp3’s PYD + NBD
Nlfp8’s PYD + NBD

(D) Vector
Hck-C1
Lyn-C1
Lck-C1
Vector
Hck-C1
Blk-C1
Fgr-C1
Src-C1
Yes-C1
Fyn-C1

2DO
4DO
4DO + 3AT

Figure 2

Nlfp12 binds to Hck
Nlrl2 binds to Hck

Figure 3
Figure 4
Nlrd12 binds to Hck

Figure 5
Nlrp12 binds to Hck

Figure 6
Nlrp12 binds to Hck

Figure 7

Spearman coefficient = 0.80
(p value < 0.001)

Pearson coefficient = 0.73
(p value < 0.001)
Figure 8

Nlrp12 binds to Hck