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MyD88 Adaptor-Like D96N Is a Naturally Occurring Loss-of-Function Variant of TIRAP

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Signals elicited by TLRs following the detection of microbes are integrated and diversified by a group of four cytoplasmic adaptor molecules featuring an evolutionarily conserved Toll/IL-1R signaling domain. Single nucleotide polymorphisms (SNPs) in TLRs and their adaptor molecules have been shown to influence susceptibility to a range of infectious and other diseases. The adaptor MyD88 adaptor-like (Mal)/Toll/IL-1R–containing adaptor protein is involved in TLR2 and 4 signal transduction by recruiting another adaptor molecule, MyD88, to the plasma membrane. In this study, we used naturally occurring variants of Mal as tools to study the molecular biology of Mal in more detail in cellular model systems and to thereby identify functionally interesting variants whose corresponding nonsynonymous SNPs might be of further epidemiological interest. Of seven reported variants for Mal, we found Mal D96N associated with reduced NF-κB signaling and cytokine production after overexpression in HEK293 and Huh-7 cells. The D96N mutation prevented Mal from recruiting its signaling partner MyD88 to the plasma membrane and altered posttranslational modification of Mal. These findings led us to investigate the frequency of heterozygosity for the corresponding SNP rs8177400 in a Caucasian case-control study on the etiology of lymphoma, a disease in which TLRs have been implicated. Although rs8177400 did not modify lymphoma risk in general, its frequency of heterozygosity was accurately determined to 0.97%. Our data add rs8177400 (D96N) to the list of functionally important variants of Mal and warrant further research into its immunological, epidemiological, and diagnostic relevance.

In mammals, innate immunity relies on TLR pathways for the detection of invading pathogens (1). TLRs are a family of germline-encoded pattern recognition receptors endowed with the capacity to recognize different pathogen-associated patterns (PAMPs) (1, 2). Upon PAMP recognition, TLRs trigger the production of proinflammatory cytokines and IFNs by transcriptional regulation and thus initiate and shape adaptive immune responses (3). Different TLRs possess specificity for diverse structural classes of pathogen molecules. For example, TLR2 is the receptor for bacterial lipopeptides and TLR4 detects bacterial LPS (1). TLRs engage PAMPs in the extracellular space or endosomes and their cytoplasmic Toll/IL-1R (TIR) domains relay signals intracellularly where they are integrated and diversified by TIR, Toll/IL-1R; WT, wild-type.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: HA, hemagglutinin; Hc, heavy chain; HEK, human embryonic kidney; IRAK, IL-1R–associated kinase; Lc, light chain; Mal, MyD88 adaptor-like; nsSNP, nonsynonymous single nucleotide polymorphism; PAMP, pathogen-associated molecular pattern; PI2P, phosphatidylinositol 4,5-bisphosphate; Pten, phosphatase and tensin homolog; SNP, single nucleotide polymorphism; TIR, Toll/IL-1R; WT, wild-type.

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Extremely rare point mutations in TIR adaptors have recently been linked to an increased susceptibility to infectious agents in affected human individuals (11). Similarly, single nucleotide polymorphisms (SNPs) in TLR signaling molecules have been associated with infectious, inflammatory, or autoimmune diseases (reviewed in Ref. 12). In Mal, rs7932766 (A186A), a variant in strong linkage disequilibrium with S180L, was recently shown to be associated with an increased susceptibility for tuberculosis in a small Vietnamese study (13), and heterozygous carriage of the nonsynonymous (amino acid-changing) Mal SNP (nsSNP), rs8177374 (S180L), was associated with substantial protection against invasive pneumococcal disease, bacteremia, malaria, and tuberculosis in multiple populations and studies involving thousands of individuals (14). S180L was found protectively associated with tuberculosis in a Colombian (15) but not in a Russian (13, 16) study and with malaria in a Brazilian study (17). An association with systematic lupus erythematosus (15) but not rheumatoid arthritis has also been reported (13, 16). Overall, increasing evidence suggests that SNPs in Mal and other TLR signaling molecules may be considered important genetic determinants for disease. Unfortunately, most studies have not addressed the functional and structural consequences of these naturally occurring, nsSNP-associated phenovariants on the molecular level.

In this study, we used naturally occurring variants as probes to study the biology of the adaptor molecule Mal in more detail, with a view of identifying variants whose corresponding nsSNPs might be of epidemiological interest due to an interesting functional phenotype.

Materials and Methods

Reagents and cells

Reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. HEK293 cells (A. Dalpé, Department of Hygiene and Medical Microbiology, University of Heidelberg, Heidelberg, Germany) were cultured in DMEM supplemented with 10% FCS, 1-γ-glutamine, and penicillin/streptomycin (all from Invitrogen, Carlsbad, CA) at 37°C and 5% CO2. Huh-7 cells (R. Bartenschlager, Department for Infectious Diseases, University of Heidelberg) were maintained in the above media supplemented with nonessential amino acids (Invitrogen), MyD88-deficient IκBα cells (G. Stark, Department of Molecular Genetics, Lerner Research Institute, Cleveland, OH) (18) were cultured as above. TLR2 and 3 ligands were from Axxora (Lörrach, Germany).

Cloning and site-directed mutagenesis

N-terminal hemagglutinin (HA); YPYDVPDYA) and myc (EEQKLISEEDL)-tag encoding oligonucleotides were inserted into pcDNA3.1(+), and subsequently, a Mal or MyD88-encoding PCR product was inserted, respectively. Template plasmids (U. Hasan, International Agency for Research on Cancer, Lyon, France) contained human Mal (235-aa isoform; accession number AF406652) and MyD88 (accession number U70451). Point mutations corresponding to Mal nsSNPs were introduced (QuickChange II Kit; Stratagene, La Jolla, CA) and verified by automated DNA sequencing. Mutated Mal inserts were back-cloned into the original backbones to avoid unwanted mutations. PCR and mutagenesis primer sequences are listed in Supplemental Table III.

Signaling assays, IL-8 ELISA, and quantitative real-time PCR

HEK293 cells in 24-well plates (7.5 × 103 cells/well) were transfected using the calcium phosphate method with a firefly luciferase reporter (100 ng; Promega, Madison, WI), a Mal or MyD88-encoding PCR product (pRl-TK, 20 ng; Promega, Madison, WI), and pc1-EGFP (BD Clontech, Palo Alto, CA) to monitor transfection, incubated for 48 h, lysed, and measured using the Dual Luciferase Assay system (Promega). Different amounts of TIR adaptors were transfected as described previously. Huh-7 cells were seeded in 24-well plates (30,000 cells/well) in media without penicillin/streptomycin, and transfected (Lipofectamine 2000; Invitrogen) 24 h later with 10 ng of plasmids (50 ng/ml NE-84-fluffy, 5 ng/ml transfected Lipofectamine luciferase control reporter, 20 ng/ml Mal scrambled reporter, and 50 ng Mal variants). Luciferase activity was measured as above.

For measuring IL-8 production, transfection was carried out as above, and IL-8 concentrations in cell supernatants were determined 48 h later using a human IL-8 ELISA kit (Biolegend, San Diego, CA). For quantitative real-time PCR, 2.25 × 104 human embryonic kidney (HEK) cells were transfected with 240 ng of the respective plasmids. Forty-eight hours later, total RNA extraction was performed (RNeasy kit; Qiagen, Valencia, CA), cDNA synthesized (Superscript III; Invitrogen), and quantitative PCR performed in duplicates using the Lightcycler 480 Probes Master Mix (Roche, Basel, Switzerland). Hypoxanthine phosphoribosyltransferase-1 was used as a reference gene. For primers and probes see Supplemental Table III.

Confocal immunofluorescence microscopy

HEK293 and Huh-7 cells seeded on poly-t-lysine–treated cover slips were transfected as above. After 40 h, cells were fixed using 2% formaldehyde and permeabilized using 1% Triton X-100 in PBS. HEK293 cells were stained with mouse anti-HA-Alexa594 (5 μg/ml; Invitrogen) and/or mouse anti–myc-Alexa 488 Ab conjugates (0.8 μg/ml; Cell Signaling Technology, Beverly, CA) in PBS. Huh-7 cells were stained with mouse anti-myc (8 μg/ml; Sigma-Aldrich) and rabbit anti-HA (1/250; Cell Signaling Technology) and subsequently with Alexa 488 anti-mouse IgG or Alexa 594 anti-rabbit IgG conjugates (2.5 μg/ml, respectively; Invitrogen). To visualize nuclei, Hoechst 33342 stain was used (2 μg/ml). Cells were preserved using Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Live cell imaging was done in Lab-Tek chamber slides 48 h posttransfection at 37°C and 5% CO2 (see above). Images taken using widefield optics for ticsasis (Cell Signaling Technology) or confocal (for analysis images and live cell imaging on a Leica SP5, ×40-63) microscopy with sequential scanning were processed using Leica LAS AF Lite or Zeiss AxioVision software.

Commmunoprecipitations and expression analysis by one-dimensional SDS-PAGE

HEK293 cells seeded in 10-cm dishes (1.5 × 106 cells/dish) were transfected as before with 5 μg TIR adaptor plasmid or empty vector (pcDNA3.1(+)) and 1 μg pc1-EGFP. Forty-eight hours later, cells were scraped in lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.05% CHAPS, and 0.5% Nonidet P-40) containing protease and phosphatase inhibitor mixtures (Roche) and lysed. Protein concentrations were determined in cleared lysates (Bichinchoninic Acid Protein Assay; Pierce Chemical Co., Rockford, IL) and adjusted to the same protein concentrations. For communoprecipitations, 800 μl cell lysates was incubated first with 2 μg of the respective Abs (1 h, 4°C), then additionally with protein A/G beads (35 μl/sample, 4 h, 4°C; Pierce Chemical Co.). Beads were washed three times with lysis buffer. For one-dimensional SDS-PAGE, beads were resuspended in NuPage loading buffer (Invitrogen) and boiled, and equal volumes were loaded per lane of 4–12% Bis-Tris gradient gels (MES buffer; Invitrogen). For expression analysis, equal protein amounts of total cell lysates were analyzed on 4–12% Bis-Tris gels per lane and protease inhibitors (Roche) and loaded onto immobilized pH gradient strips (pH 3 to 10; GE Healthcare) overnight at 30V. Isoelectric focusing (IEF) strips (GE Healthcare) were done at 300 V (60 min), followed by gel electrophoresis (PAGE) on 4–12% Bis-Tris gels (over 30 min), 1000–5000 V (over 90 min), and 5000 V (30 min). Strips equilibrated in LDS buffer (Invitrogen) with 1% DTT (15 min) and 2.5% Iodoacetamide (15 min) were mounted onto NuPage 4–12% Bis-Tris ZOOM gels (Invitrogen) for second dimension separation (MES buffer; Invitrogen). After semidry transfer, polyvinylidene difluoride membranes (Millipore, Bedford, MA) were probed (overnight at 4°C) using mouse anti–myc (0.4 μg/ml; Cell Signaling Technology) or anti–β-actin (0.4 μg/ml; Cell Signaling Technology) or anti-phospho-tyrosine clone 4G10 (1 μg/ml in 5% BSA in TBS-0.1% Tween; Millipore), followed by anti-mouse HRP conjugates (Promega).

Sequence alignments, homology modeling, and structural analysis

Protein sequences were from Swiss-Prot (www.expasy.org), and alignments were color-formatted according to amino acid physicochemical properties (Joy server: tardis.nibio.go.jp). Homology modeling was based on the
MyD88 TIR domain nuclear magnetic resonance structure (Protein Data Bank code: 2j57, Ref. 19). Sequence alignments were generated using ClustalX, manually optimized and subjected to Modeler (20). GROMACS, ANOLEA, VERIFY_3D, and ERRAT were used to improve and score models for energy and sterical correctness (Table Supplement II), respectively. Pymol and Adaptive Poisson-Boltzmann Server were used for structure analysis and surface charge calculations (see Ref. 21 for software citations).

Study population: German multicenter case-control study on the etiology of lymphomas

A population-based case-control study approved by the local ethical committees was carried out from 1999 to 2002 in six regions of Germany among 18- to 80-y-old adults, 1:1 matched for gender, age (± 1 y of birth), and study region (for details, see citations in Ref. 22). The study comprises 710 case-control pairs, including 554 cases of B-non-Hodgkin’s lymphoma, 158 cases of diffuse large B cell lymphoma, 92 cases of follicular lymphoma, 104 cases of B cell chronic lymphocytic leukemia, 29 cases of multiple myeloma, 21 cases of T cell chronic lymphocytic leukemia, 15 cases of T non-Hodgkin’s lymphoma, and 115 cases of Hodgkin’s lymphoma. One case was diagnosed as Hodgkin’s lymphoma and non-Hodgkin’s lymphoma. Controls were drawn randomly from population registers of the study regions. Written informed consent was obtained from each subject.

Polymorphism information, analysis and genotyping

A list of reported SNPs in human TIRAP (Gene ID: 114609), was obtained from National Center for Biotechnology Information at www.ncbi.nlm.nih.gov (Supplemental Table I). HapMap data were from www.hapmap.org (23). Genotyping was performed by pyrosequencing as described previously (22). PCR mixtures contained: 5 ng DNA, 1 × Ready Mix PCR buffer (ABgene, Epsom, U.K.), 0.25 U of Thermoprime DNA polymerase (ABgene), deoxynucleoside triphosphates, each at 167 μM (PeqLab, Erlangen, Germany), and primers (3 pmol each) in a total reaction volume of 12 μl. Cycling conditions 36 cycles, 94°C 40 s, 57°C 40 s, and 72°C 40 s. For primers, see Supplemental Table III.

Statistical analysis of association study

For signaling assays, p values were determined using the Student t test; *p < 0.01 and **p < 0.05. For genotyping studies, unconditional logistic regression models adjusted for age, sex, and study center were used to obtain odds ratios as estimates of relative risk. Genotype was coded as an ordinal variable based on the number of rare alleles (0, 1, 2) and as a binary variable assuming dominant inheritance. The reference group for genotype was WT homozygous genotype in controls. Analysis was preformed with SAS version 9.0.

Results

Mal D96N is a loss-of-function variant of TIRAP

Of >100 SNPs reported in the human TIRAP gene region on chromosome 11 (Location: 11q24.2), 7 nsSNPs map to its coding region and lead to an amino acid exchange in the Mal protein (Supplemental Table I): A9P and R13W (N-terminal domain), S55N (PEST region), D96N, S180L, V197I (TIR domain), and X222W, which converts a STOP codon into a tryptophan codon, leading to a longer isoform of Mal (Fig. 1A). We considered these naturally occurring variants as interesting probes to gain insights into the biology of Mal and concentrated on the six reported nsSNPs leading to point mutations (X222W was not investigated in this study). Initially, amino acid conservation in the location of these SNPs was analyzed by carrying out sequence alignments of Mal protein orthologs from human to Xenopus (Supplemental Fig. 1). We found that all six amino acids in question are highly conserved in mammals. Mal D96 is even conserved from humans to nonmammalian species, such as chicken and Xenopus. We considered that it may be of high functional or structural importance.

To study the naturally occurring variants of Mal functionally, corresponding expression constructs were generated. Mal P125H was included in some experiments for comparison (7). Although D96N and P125H expressed at slightly lower levels, other myc-tagged mutant constructs expressed similarly to WT Mal (Fig. 1, A and B). We found that all six amino acids in question are highly conserved in mammals. Mal D96 is even conserved from humans to nonmammalian species, such as chicken and Xenopus. These results suggest that it may be of high functional or structural importance.

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Mal D96N IS A LOSS-OF-FUNCTION VARIANT OF TIRAP

1B). We next investigated whether overexpression of the Mal variants would lead to NF-κB activation on a level similar to WT by dual luciferase reporter assays in HEK293 cells. As shown in Fig. 1C, most Mal variants reproducibly exhibited levels of NF-κB reporter activation comparable to WT (S55N, S180L, and V197I) or slightly attenuated (A9P and R13W). Interestingly, NF-κB activation was completely abolished for Mal D96N. We also measured the production of secreted IL-8 from Mal-transfected cell cultures by ELISA. Similarly to NF-κB activation, most mutants produced IL-8 to a similar level as Mal WT as a consequence of Mal overexpression (Fig. 1D). Again, the R13W mutant was showing a reduction in IL-8 production. Most strikingly, IL-8 production was severely diminished for D96N, suggesting that this variant is impaired in downstream signaling. This was also confirmed at the level of IL-8 and TNF-α mRNA induction as analyzed by quantitative real-time PCR (Fig. 1E). We consistently observed signaling comparable to WT for Mal S180L (cf. Ref. 14) in all assays in the HEK293 system (Fig. 1C–E). Similar results were obtained for D96N and S180L in Mal signaling assays in Huh-7 cells (Fig. 1F). To investigate whether D96N could influence the ability of WT Mal protein to signal, we performed NF-κB dual luciferase assays and measured IL-8 production by ELISA in HEK293 cells cotransfected with increasing amounts of D96N and a constant amount of WT Mal. Mal P125H was used as a dominant-negative control (7). As shown in Supplemental Fig. 2A and 2B, increasing plasmid amounts of D96N moderately but dose-dependently decreased NF-κB activity and secreted IL-8 levels, indicating that Mal D96N may modulate Mal-dependent signaling pathways if expressed in excess. In contrast, TLR2-dependent NF-κB signaling, which relies on Mal, was not markedly affected in the HEK293 system (Supplemental Fig. 2C, 2D). In the following, we focused our studies on the D96N variant. Considering lower expression levels an incomplete explanation, we conducted a series of functional and structural analyses to investigate the molecular basis for the observed inability of D96N to initiate NF-κB signaling.

Plasma membrane trafficking and recruitment of MyD88 are impaired for Mal D96N

We initially hypothesized whether the ability of Mal to engage in signaling complexes could be compromised in Mal D96N. Mal signaling as a result of overexpression is strictly MyD88 dependent as confirmed by experiments in MyD88-deficient HEK293 cells (18) (data not shown). In normal HEK293 cells, Mal overexpression leads to NF-κB activation. We therefore concentrated on signaling events downstream of TLR2 and TLR4 and initially analyzed the ability of Mal to self-associate as shown earlier (10). We cotransfected HA-Mal WT or D96N and myc-Mal WT or D96N into HEK293 cells and analyzed protein complexes by immunoprecipitation and immunoblot. As evident from Fig. 2A, Mal D96N was able to interact with WT Mal and WT Mal, suggesting that oligomer formation was not compromised. Co-transfecting HA-Mal WT or D96N and myc-MyD88, we investigated next whether Mal D96N could still engage its signaling partner MyD88 as reported previously for WT Mal (7, 10). Fig. 2B illustrates that interactions for Mal D96N with MyD88 were similar to Mal WT. Because D96N appeared not to affect molecular interactions of Mal, we sought to address by immunofluorescence confocal microscopy whether Mal D96N trafficked differently to WT in transfected HEK293 cells. Fig. 2C shows staining for myc-Mal WT at the plasma membrane, in addition to some cytoplasmic staining. In Mal D96N-transfected cells, cytoplasmic staining was also visible and appeared more (condensed) punctate. Compared with WT Mal, staining along the plasma membrane was less pronounced for Mal D96N. This was verified using z-stack analysis of individual cells (data not shown). In cotransfections of both myc-Mal WT and HA-D96N, the two proteins partially colocalized intracellularly and (to a lesser degree) at the plasma membrane (Fig. 2D and insets), which is in keeping with our communoprecipitation studies. We next investigated the redistribution of overexpressed MyD88 from distinct cytoplasmic foci to a more uniform intracellular distribution and localization to the plasma membrane, which was observed as a result of Mal coexpression (8). As Fig. 2E shows, in the majority of cells (>65%), MyD88 located to cytoplasmic foci in the absence of Mal but was redistributed across the cytoplasm and membrane-proximally as expected when Mal WT was coexpressed (Fig. 2F). In membrane-proximal areas, myc-Mal and HA-MyD88 partially colocalized. When D96N was expressed (Fig. 2G), MyD88 showed the same focal staining observed in single transfections of MyD88 (Fig. 2E) and was not redistributed across the cytoplasm or to the plasma membrane. At the observed cytoplasmic focus, MyD88 and Mal D96N showed colocalization (as verified by two-dimensional scatter plot analysis), suggesting that the interaction shown earlier (Fig. 2B) occurs at these sites. This was confirmed by analysis of wide field tilescans (data not shown) and in Huh-7 using both Ab staining in fixed cells (Fig. 2E–G) and live cell imaging (Fig. 2H).

In conclusion, our data therefore suggest that although Mal D96N and MyD88 physically interact, this complex is unable to reach the plasma membrane.

D96 influences posttranslational modification of Mal

To further assess the structural basis for the inability of D96N Mal to signal, we generated three-dimensional homology models of the Mal WT and D96N TIR domains based on the nuclear magnetic resonance structure of the MyD88 TIR domain (19) and subjected it to a 10-ns molecular dynamics simulation (Materials and Methods). Tertiary structure and surface charge were subsequently analyzed in both models. Fig. 3A shows that the structures of Mal WT and D96N are relatively similar with regard to their tertiary structure but differ in the spatial arrangement of several flexible loops (particularly EE loop) that connect the main secondary structure elements. Several loops (BB, AA, and CC) meet at a surface region in which residue 96 is located in the AA loop of the molecule in proximity to the BB loop (Fig. 3B and Supplemental Fig. 3). The WT model features a highly negatively charged surface formed by D96, two adjacent glutamic acid residues, E94 and E95 (AA loop), D102 (αA), and D122 (BB loop). This charged patch is much less pronounced in the D96N model (Fig. 3A). To investigate whether this effect on surface charge could contribute to the observed loss of function, we mutated aspartic acid 96 to glutamine (a residue equivalent to asparagine in D96N, therefore expected to result in charge reversal like the D96N amino acid exchange), arginine (to alternatively reverse charge), or glutamic acid (the equivalent of aspartic acid to maintain a negative charge; predicted in Fig 3B). The expression of these charge-affecting mutants was verified by immunoblot (Fig. 3C) and their signaling properties by NF-κB dual luciferase assay. Fig. 3D shows that, expectedly, D96Q and D96R were completely defective in signaling, which would unlikely be accounted for by the observed slight differences in expression level. Surprisingly, D96E, a conservative substitution predicted to maintain the negative charge, also resulted in complete loss of function and the charge introduced by the gamma-carboxyl group of glutamate appeared not to restore signaling to WT levels. We wondered whether the low tolerance for substitution at position 96 hinted to a more distinct functional role for D96 and whether its mutation might affect reported posttranslational modifications of
Mal that could regulate its activity and/or trafficking (5). Therefore immunoprecipitates from myc-tagged WT, D96N-, and D96E-transfected cells were analyzed by two-dimensional gel electrophoresis combined with immunoblot. The myc-Mal WT blot (Fig. 3E, panel 1) repeatedly (∼5) showed that Mal WT existed as a range of different species (cf. orange signals in panels 4 and 5), which is indicative of posttranslational modification. In the D96N blot (Fig. 3E, panel 2), several spots observed for Mal WT were clearly absent (marked by asterisks in panel 5). This was not linked to lower expression of D96N and was also observed for D96E (data not shown). When probing for Mal tyrosine phosphorylation (9), we observed clear differences in the number and relative abundance of different species (Fig. 3F). This strongly suggested that mutations at D96 influence the posttranslational modification of Mal.

Mal D96N occurs heterozygously in ∼1% of Europeans

Because our data showed that the reported naturally occurring variant D96N in human TIRAP results in a loss of function in the Mal protein, we sought to obtain a first impression of the potential epidemiological relevance of the corresponding SNP rs8177400. As polymorphisms in several TLRs have been associated with certain subtypes of lymphoma (22, 24), we decided to genotype rs8177400 in 665 cases and 678 age, sex, and area matched population-based controls of a German case-control study on the etiology of lymphoma (22). We found 13 heterozygous individuals —6 (0.9%) in controls and 7 (1.03%) in lymphoma patients. Among the patients, heterozygotes were found in 1 of 9 precursor B cell non-Hodgkin’s lymphoma, 2 of 101 follicular lymphoma, 2 of 104 chronic lymphocytic leukemia, 1 of 105 Hodgkin’s lymphoma, and 1 of 72 other B cell non-Hodgkin’s lymphoma patients. No homozygous A/A genotypes were detected. Overall the frequency of heterozygosity for rs8177400 was 0.97% in this Caucasian study population. Lymphoma risk in general was not modified in association with rs8177400, although statistical power was limited.

Discussion

In this study, we sought to address the question whether known naturally occurring variants of Mal influence the function of this important signaling adaptor, and if so, which changes on the molecular level were responsible for the observed phenotype. Our
Mal WT but not D96N samples are denoted by asterisks. In D96N (marked, in parentheses) or 0 (control) or 5 or 40 ng plasmid DNA for each D96 mutant construct, and their signaling properties were assessed by NF-κB activation and cytokine mRNA induction and secretion (Fig. 1). The data we obtained for D96N in Huh-7 cells, as assessed by NF-κB dual luciferase assay. One of three experiments is shown (triplicate means + error). C, Mal D96 mutants are expressed in transfected HEK293 cells. Cell lysates of transfected cells were separated and probed using anti-myc (upper panel; loading control) and anti-β-tubulin (lower panel; loading control) Abs. D, Different mutations at position 96 render Mal dysfunctional. HEK293 cells were transfected with 0 (control) or 5 or 40 ng plasmid DNA for each D96 mutant construct, and their signaling properties were assessed by NF-κB dual luciferase assay. One of three experiments is shown (triplicate means + error). E, The D96N mutation influences posttranslational modification in Mal. myc-Mal WT (panel 1) and D96N (panel 2) immunoprecipitates from HEK293 cells were analyzed by two-dimensional gel electrophoresis and blotted, and membranes were probed with anti-myc (E, one of five experiments) or anti-phosphotyrosine (F, one of three experiments) Abs. Signals for Mal, heavy (Hc), and light (Lc) chains are marked. In panels 4 and 5, signals from Mal WT (orange) and D96N (blue) were overlaid. Signals found in both blots appear black; those only occurring in Mal WT but not D96N samples are denoted by asterisks. In F, acidic species were numbered (see Results).

FIGURE 3. D96 influences posttranslational modification of Mal. A, Upper panels, Homology model of the Mal WT (light blue) and D96N (gray) TIR domain. Secondary structure elements are as follows: α-helices are light blue, β-strands are dark blue, and connecting loops are orange. The αD helix is disordered. Lower panels, Surface charge predictions for additional mutations at position 96: glutamine (equivalent to asparagine), arginine (to reverse charge similar to asparagine), and glutamic acid (to maintain a negative surface charge). B, Mal D96 mutants are expressed in transfected HEK293 cells. Cell lysates of transfected cells were separated and probed using anti-myc (upper panel) and anti-β-tubulin (lower panel; loading control) Abs. D, Different mutations at position 96 render Mal dysfunctional. HEK293 cells were transfected with 0 (control) or 5 or 40 ng plasmid DNA for each D96 mutant construct, and their signaling properties were assessed by NF-κB dual luciferase assay. One of three experiments is shown (triplicate means + error). E, The D96N mutation influences posttranslational modification in Mal. myc-Mal WT (panel 1) and D96N (panel 2) immunoprecipitates from HEK293 cells were analyzed by two-dimensional gel electrophoresis and blotted, and membranes were probed with anti-myc (E, one of five experiments) or anti-phosphotyrosine (F, one of three experiments) Abs. Signals for Mal, heavy (Hc), and light (Lc) chains are marked. In panels 4 and 5, signals from Mal WT (orange) and D96N (blue) were overlaid. Signals found in both blots appear black; those only occurring in Mal WT but not D96N samples are denoted by asterisks. In F, acidic species were numbered (see Results).
phosphorylation events in the PTEN C terminus modulate PIP2 with its C terminus (27). In fact, in PTEN the negatively charged surface of lipid membranes (8). D96 is the interaction of the N-terminal PIP2 motif in phosphatase and directly contact the Mal N terminus. This would be reminiscent of that remain elusive. Alternatively, it is also conceivable that D96 complexes may be able to enter the appropriate trafficking routes terminants. Potentially only correctly modified Mal-MyD88 which are influenced by residues such as D96, may be such de-recruitment toward the membrane. Posttranslational modifications, thus also seem to influence Mal trafficking and MyD88 recruitment toward the membrane. 

In the HEK293 system, TLR2-dependent signaling was unaffected by D96N and MalWT-induced signaling only at a molar excess of D96N (Supplemental Fig. 2). Our data tentatively suggest that, depending on their relative abundance, Mal D96N may be able to modulate Mal WT-dependent signaling, a question requiring clarification in cells isolated form D96N carriers. Similar studies proved insightful in patients carrying rare MyD88 (11) or IRAK-4 (28) mutations. Whereas mutations resulting in no or subtle functional consequences may escape negative selective pressure (e.g., TLR4 D299G, T399I; Ref. 29, mutations that severely compromise function would probably not be maintained in a population exposed to microbial pathogens, unless heterozygosity resulted in a protective phenotype). D96N rs8177400 G722 A had been found heterozygously in small African-American (0 of 44; 0%) and European (2 of 46; 4.3%) collections, and the HapMap “Tuscans in Italy” population (1 of 77; 1.2%) and “Han Chinese in Beijing” (1 of 82; 1.2%) populations (Supplemental Table 1 and Ref. 23) but was not polymorphic in a small Vietnamese study population (n = 45; Ref. 13). Our genotyping studies confirm previous preliminary studies and demonstrate that the frequency of heterozygosity of D96N is 0.97% in a Caucasian population. Further preliminary studies in other ethnic groups show that Mal rs8177400 (D96N) also occurs in non-Caucasians with low (<1%) frequency (A. Rautanen, T.C. Mills, A. V.S. Hill, and A.N.R. Weber, unpublished data) in keeping with HapMap data (23). These studies would need to be extended to determine the frequency of this nsSNP in non-Caucasian populations more accurately. Our analyses on the influence of rs8177400 (D96N) on the etiology of lymphoma remain inconclusive because of low statistical power, but the combined epidemiological and functional data warrant the inclusion of rs8177400 into future genetic association studies with larger sample sizes. Focusing such studies on already functionally characterized nsSNPs such as rs8177400 (D96N), rs8177374 (S180L), or rs8177399 (R13W) may not only prove fruitful for unraveling further genetic associations with disease but also cost-effective. Further studies systematically investigating the relevance of naturally occurring mutations in other adaptors or pattern recognition pathways may prove insightful both to understand the molecular biology of these molecules more fully as well as to uncover further genetic variants that may profoundly affect disease outcome.

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Disclosures
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References


