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A Naturally Occurring Variant in Human *TLR9*, P99L, Is Associated with Loss of CpG Oligonucleotide Responsiveness[S]

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The innate immune system employs Toll-like receptors (TLRs) for the detection of invading microorganisms based on distinct molecular patterns. For example, TLR9 is activated by microbial DNA and also by short therapeutic CpG-containing oligonucleotides (CpG-ODN). TLR9 activation leads to the production of interferons and the priming of humoral adaptive immune responses. Unfortunately, the principles of ligand recognition by TLR9 are poorly understood, and genetic variants of TLR9, which may affect its function, have not been characterized systematically on the molecular level. We therefore sought to functionally characterize reported single nucleotide polymorphisms of TLR9 in the HEK293 model system. We discovered that two variants, P99L and M400I, are associated with altered receptor function regarding NF-κB activation and cytokine induction. Our investigations show that for the most functionally impaired variant, P99L, the ability to respond to physiological and therapeutic TLR9 ligands is severely compromised. However, CpG-ODN binding is normal. CpG-ODN recognition by TLR9 thus appears to involve two separate events, CpG-ODN binding and sensing. Our studies highlight Pro-99 as a residue important for the latter process. In genotyping studies, we confirmed that both M400I (rs41308230) and P99L (rs5743844) are relatively rare variants of *TLR9*. Our data add rs41308230 and rs5743844 to the list of functionally important TLR variants and warrant further research into their relevance for infectious disease susceptibility or responsiveness to CpG-ODN-based therapies.

The detection of invading micro-organisms by vertebrates involves Toll-like receptors (TLRs),[8] a family of evolutionarily conserved pattern recognition receptors. TLRs are endowed with the capacity to recognize diverse structural classes of pathogen molecules (1). For example, TLR2 is the receptor for bacterial lipopeptides, TLR4 detects bacterial lipopolysaccharide, and TLR9 senses bacterial and viral nucleic acids containing CpG motifs, respectively (1, 2). TLR9 engages ligands present in endosomes by its extracellular domain (ECD), and its cytoplasmic Toll/IL-1 receptor (TIR) domain relays an intracellular signal via the TIR-containing adaptor molecule MyD88 (3, 4). TLR9-MyD88-dependent signaling involves the recruitment and activation of IL-1 receptor-associated kinase-4, IL-1 receptor-associated kinase-1, and TNF receptor-associated factor-6. This ultimately leads to the activation of the NF-κB and AP-1 transcription factors and the production of proinflammatory cytokines in several cell types. In plasmacytoid dendritic cells, the MyD88-dependent activation of the IRF7 transcription factor drives cell maturation and differentiation and leads to the production of type I IFN (4, 5). In B lymphocytes, cellular outcomes of TLR9 ligation are antigen-specific stimulation and enhanced immunoglobulin G class switch. Both are required for the establishment of a sufficient humoral immune response (5). On the other hand, TLR9-dependent activation of self-reactive B cells has recently been recognized as a contributing factor in autoimmune diseases (6). The cellular effects of TLR9 activation in plasmacytoid dendritic cells and B cells are recapitulated upon stimulation with synthetic CpG motif-containing oligonucleotides (CpG-ODN) and is now widely recognized that CpG-ODN represent promising therapeutics able to initiate and shape adaptive immune responses (5).

The TLR9 ECD consists of 19–25 tandem arrangements of the leucine-rich repeat (LRR) motif, which together form a horseshoe-shaped solenoid that is capped by cysteine-rich regions. The first ~10 LRR residues are relatively conserved, adopt a β-sheet conformation and form the concave surface of the ECD, whereas the remaining portion of each LRR is more

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[8] The abbreviations used are: TLR, Toll-like receptor; ECD, extracellular domain; LRR, leucine-rich repeat; ODN, oligodeoxynucleotide; TIR, Toll/IL-1 receptor.

variable and contributes to the convex surface (3). All TLRs, including TLR9, contain varying numbers of "irregular" LRRs that deviate from the consensus motif and bear inserting stretches of amino acids thought to protrude from the horseshoe-shaped backbone (7). In TLR8 and -9, these insertions were recently shown to play an important role for receptor function (8–10). Whereas ligand engagement by TLR2 and TLR4 follows a different structural framework (11, 12), nucleic acid sensing by TLRs appears to involve N- and C-terminal charged binding patches as recently shown by crystallographic and mutagenesis studies for the RNA-sensing TLR3 and for murine TLR9, respectively (10, 13). Conflicting data exist with regard to the notion that N-terminal cleavage of the TLR9 ectodomain by endosomal proteases is necessary to yield an active receptor (discussed in Ref. 10).

Extremely rare point mutations or single-nucleotide polymorphisms (SNPs) in TLRs and TIR adaptors have recently been linked to altered susceptibility to infectious agents in affected human individuals (14–17). The profile of currently known genetic variants in TLR9 and other nucleic acid-sensing TLRs has been proposed to associate with severe clinical phenotypes (18). Due to its functional role in both infectious and autoimmune diseases, TLR9 genetic variants have been investigated in the susceptibility for asthma (19, 20), HIV (21, 22), malaria, and systemic lupus erythematosus (23–26). Unfortunately, so far, the functional and structural consequences of SNP-associated TLR9 phenovariants have not been investigated systematically on the molecular level. In this study, we sought to address whether reported sequence variants of TLR9 would be associated with altered receptor function in a model cellular system to identify variants with potential epidemiological significance.

EXPERIMENTAL PROCEDURES

Reagents and Cells—Chemicals and cell culture reagents were from Sigma, unless otherwise stated. HEK293 cells were a gift from A. Dalpke (University of Heidelberg) and were cultured in DMEM supplemented with 10% fetal calf serum (PAA Laboratories), L-glutamine, and penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO_2. The CpG phosphorothioate ODN 2006 (5′-tcgtcgttttgtcgttttgtcgtt-3′) was synthesized by TIB MOLBIOL. Other ODN were from GATC Biotech. The following antibodies were used: anti-HA (Sigma and Cell Signaling), anti-GFP (Sigma), anti-TLR9 (eBioscience), anti-Calreticulin (BioReagents), anti-β-tubulin (Sigma), anti-rabbit HRP (Vector Laboratories), and anti-mouse HRP (Promega).

Plasmids and Site-directed Mutagenesis—A plasmid containing the TLR9-coding sequence (NCBI accession nos. NT_022517.18/NP_059138.1) was described earlier (27). hTLR9-HA plasmids were constructed by introducing an annealed custom synthesized 5′-phosphorylated ODN encoding HA (YPYDVPDYA) tag sequence using the restriction enzymes BamHI and NotI. hTLR9-YFP or -YPet (a gift from P. Daugherty, Department of Chemical Engineering University of California, Santa Barbara, CA) (28) was constructed by ligating a PCR fragment corresponding to the YFP or YPet coding sequence using BamHI and NotI restriction. All tagged versions of TLR9 were verified to be responsive to CpG-ODN (data not shown). Site-directed mutagenesis was carried out as described earlier (10); sequences of all oligonucleotides are available upon request.

Reporter Gene Experiments and Real-time PCR—For reporter gene experiments, a firefly luciferase reporter construct with a 6× NF-κB responsive element was used. 1×10^5 HEK293 cells were seeded and immediately transfected in 24-well format and a volume of 500 μl of media. 50 ng of hTLR9-HA or the indicated mutant plasmids was transfected with 85 ng NF-κB reporter plasmid encoding firefly luciferase and 8.5 ng pRL-TK (Promega) encoding *Renilla* luciferase were transfected using the calcium phosphate method. The total amount of TLR9 plasmids was kept to 50 ng to avoid an increase in background activation. 24 h after transfection, cells were stimulated with 1 μM or indicated concentrations CpG-ODN 2006 for 18 h, and luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega) on a Fluostar Optima Instrument (BMG Labtech). Values were normalized to unstimulated (media only) values for each transfection. Mean values of triplicates (± S.D.) of one out of at least three independent experiments are shown. For real-time PCR experiments, cells were harvested as above in RLT buffer, and RNA was extracted using the RNeasy kit (Qiagen). Per sample, 1 μg RNA was reverse-transcribed (Superscript III, Invitrogen) in 20 μl using oligo(dT) primers (Promega), and 1 μl of this reaction was subsequently used per well in a real-time PCR reaction using the Universal Probe Library (Roche) hybridization probes on a LightCycler 480 instrument. Primers are listed in supplemental Table S2, and Universal probes used are as follows: IL-8 (probe no. 72), TNF-α (probe no. 40), and hypoxanthine phosphoribosyltransferase-1 (probe no. 73). RT-minus controls were negative. Data were analyzed on LightCycler software, and inductions were calculated relative to hypoxanthine phosphoribosyltransferase-1. Shown is one of two representative experiments with triplicates + S.D.

Confocal Immunofluorescence Microscopy—HEK293 cells seeded on poly-L-lysine-treated coverslips were transfected as above. After 40 h, cells were fixed using 2% formaldehyde and permeabilized using 1% Triton in PBS. For hTLR9 WT mutant colocalization experiments, cells were stained with mouse anti-HA Alexa 594 (Invitrogen, 5 μg/ml) in PBS. For hTLR9 calreticulin colocalization experiments, cells were stained with rat anti-TLR9 antibody (eBioscience, 1 μg/ml) and anti-rat Alexa 488, as well as rabbit anti-calreticulin antibody (BioReagents, 1 μg/ml) and donkey anti-rabbit-Alexa 594 (Invitrogen, 2.5 μg/ml). To visualize nuclei, Hoechst 33342 stain was used (2 μg/ml, Invitrogen). Cells were preserved using Fluoromount G (Southern Biotech) and analyzed on a Leica SP5 confocal microscope using sequential scanning. Images were processed using Leica LAS AF Lite software. Further settings are available upon request.

Immunoblot—HEK293 cells were transfected as above with 400 ng of the indicated hTLR9-HA plasmid. 48 h later, cells were lysed for 30 min on ice in 100 μl lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with Complete protease inhibitor mixture (Roche)) per well, and three wells were pooled. Lysates were cleared by centrifugation at 4 °C for 15 min at 11,000 × *g*. Equal amounts of lysates were fractionated

on 3–8% Tris acetate SDS-PAGE (Invitrogen) gels and transferred to nitrocellulose membranes by wet transfer (Invitrogen). The membranes were blocked with PBS supplemented with 3% nonfat dry milk and 0.5% Tween 20, probed with mouse anti-HA (Cell Signaling, 1:4500) and anti-mouse HRP conjugate (Promega, 1:10,000), or rabbit anti-HA (Sigma, 1:2500), and anti rabbit-HRP conjugate (Vector Laboratories, 1:10,000), and cross-reactive bands were visualized using enhanced chemiluminescence (Pierce) on an Agfa automated developer.

CpG Pulldown—HEK293 cells were transfected with TLR9-HA WT or the indicated mutant plasmid and lysed as described above. Pulldown assays were performed as described earlier (10). In brief, lysates were incubated with 10 μM 3′-labeled biotin-CpG-ODN at 4 °C for 2 h. Subsequently, 30 μl of streptavidin-agarose beads were added for 2.5 h. Beads were washed three times in lysis buffer, and eluted proteins were used for Western blot analysis. HRP-labeled antibodies were visualized by ECL (GE Healthcare) using the Chemi-Smart 2000 video documentation system from PEQLAB in the automated mode to avoid overexposure and saturation of the signal. Quantification of the detected signals was done using the Bio-1D advanced software (PEQLAB). The amount of precipitated TLR9 was normalized to the amount of receptor in the lysate and compared with the TLR9 WT pulldown. In Fig. 3*C*, relative (to WT) intensities were plotted for one of three representative experiments. In Fig. 3*D*, relative NF-κB activation data (supplemental Fig. S5) divided by relative binding data (Fig. 3*C*) from one of three representative experiments, respectively, were plotted. 3′-Biotin-CpG-ODN induced NF-κB signaling identically to unlabeled CpG-ODN (data not shown).

Coimmunoprecipitations—HEK293 cells were seeded, transfected, and lysed as described above for immunoblot analysis. For one condition, six wells were pooled. Protein concentrations were determined in cleared lysates (BCA Protein Assay, Pierce) and adjusted to the same protein concentrations. 800 μl of cell lysates were incubated first with 2 μg of the respective antibodies (1 h at 4 °C) and then additionally with protein A/G beads (Pierce; 35 μl/sample for 4 h at 4 °C). Beads were washed three times with lysis buffer, resuspended in NuPage loading buffer (Invitrogen), and boiled, and equal volumes were analyzed as above.

Sequence and Structural Files for Homology Modeling—For homology modeling, the hTLR9 (NP_059138) sequence was used as described previously (10, 29) using the MODELLER package (30). The hTLR9 ECD was modeled on the hTLR3 ECD structures Protein Data Bank codes 2a0z (31) and 1ziw (32), the hTLR9 TIR domain on the hTLR1 TIR structure (33). GROMACS molecular dynamics, quality analysis tools (ANOLEA, VERIFY_3D and ERRAT), *N*-glycan analysis (GlyProt server), visualization/analysis tools (SwissPBD Viewer and PyMOL), and software for the computation of surface charges were employed as described in Refs. 10 and 29 and references therein.

Study Populations and Genotyping—Healthy blood donors of Caucasian origin ($n = 235$) were recruited from the Institute for Clinical Chemistry (Mannheim, Germany) upon informed consent. Genotyping analysis was done via Pyrosequencing in a PSQ96 MA using the Pyro Gold Reagents (Biotage) and specific primers for the functionally relevant TLR9 SNPs (see supplemental Table S2). For studies into associations with infectious disease, the Caucasian control group including healthy adult blood donors and cord blood samples from the United Kingdom (UK) and the UK Caucasian invasive pneumococcal disease (defined by the isolation of *Streptococcus pneumoniae* from a normally sterile site) sample collection has been described previously (34). The bacteraemia case control collection comprising Kenyan children has also been described elsewhere (35). The leprosy sample collection includes leprosy patients and controls recruited from the School of Tropical Medicine in Kolkata, India.

Immortalized B Cells—Immortalized B cells transformed by Epstein-Barr virus infection were obtained together with respective genomic DNA samples from the Coriell Cell Repository (Coriell Institute for Medical Research). The samples belonged to CEPH/UTAH pedigree 1408 and were GM10830 (CEU individual in HapMap), GM12147–153 and GM12157 (children of GM10830). P99L carriage was confirmed in GM10830, GM12149, and GM12150 by restriction fragment length polymorphism digest (below). Cells were cultured at 37 °C and 5% CO_2. Following serum starvation for up to 24 h to reduce basal activation, CpG stimulation was carried out for 6 or 21 h and proliferation monitored by [^{3}H]thymidine incorporation as described (36).

Polymorphism Information, Analysis, and Genotyping—A list of reported SNPs in the human *TLR9* gene (Gene ID 54106), was obtained from NCBI (www.ncbi.nlm.nih.gov) and summarized in supplemental Table S1. HapMap data were from the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) (37). P99L (rs5743844) was genotyped using BslI (New England Biolabs) restriction enzyme digestion after PCR, whereas genotyping of M400I (rs41308230) was performed using the Sequenom iPLEX assay (Sequenom, Inc.) under standard conditions. PCR conditions for amplifying sequences including rs5743844 were as follows: 95 °C for 15 min and then 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, followed by 72 °C for 10 min. The primer sequences are shown in supplemental Table S2.

RESULTS

Two Naturally Occurring Variants in TLR9 Affect NF-κB Activation in Response to CpG-ODN—Due to their potentially high impact on the outcome of infections or TLR9 therapeutics, we searched databases for reported SNPs in the human *TLR9* gene (Gene ID 54106; chromosome 3p21.3). At the beginning of this study, we found that of 50 SNPs in the *TLR9* gene region, eight affect the TLR9 amino acid sequence (nonsynonymous SNPs; see supplemental Table S1 and Fig. 1*A*): R5C, H79Q, P99L, M400I, R546Q, G629S (extracellular domain), R863Q, and A882T (TIR domain). Note that two frame-shifting and four coding (M58R, P123S, R962C, and G975C) SNPs only recently reported in the databases were not included in this analysis.

To gain an initial insight into the potential effects of the eight first reported amino acid changes, we analyzed for each SNP the level of sequence conservation by performing sequence align-

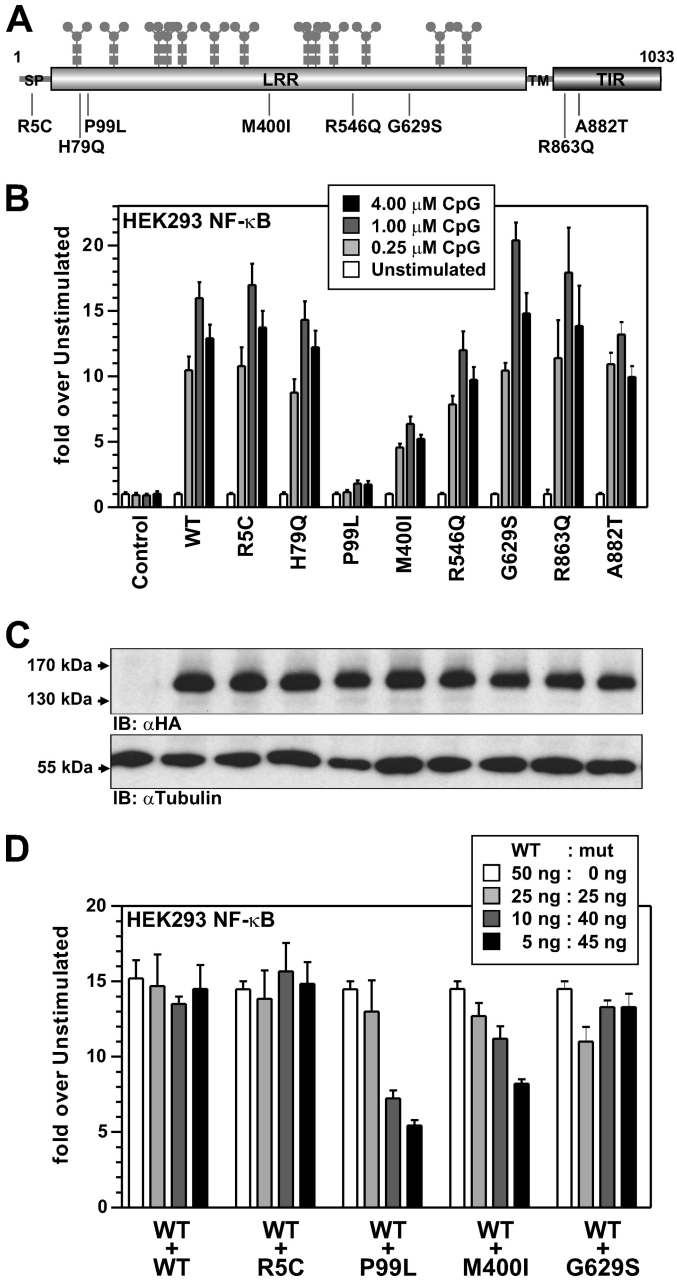

FIGURE 1. **Systematic analysis of TLR9 variants in a model system identifies P99L as a loss-of-function variant.** *A*, schematic representation of TLR9 coding sequence with glycosylation sites and location of variants indicated. *SP*, signal peptide. *B*, modulation of signaling for P99L and M400I. HEK293 cells were transfected with WT or mutant TLR9-HA constructs, stimulated with different concentrations of CpG-ODN 2006 and NF-κB activation measured by Dual-Luciferase assay. One of three independent experiments is shown (triplicates + S.D.). *C*, TLR9 mutants express to similar levels as WT. HEK293 cells were transfected with WT and mutant HA-tagged TLR9 constructs, lysed, and analyzed by anti-HA and anti-tubulin immunoblot. *D*, dominant-negative effect of P99L and M400I. HEK293 cells were transfected with different ratios of WT and mutant in a total of 50 ng of receptor DNA, stimulated with CpG-ODN 2006, and NF-κB activation was measured by Dual-Luciferase assay. One of three independent experiments is shown (triplicates + S.D.). *IB*, immunoblot.

ments of TLR9 orthologs. Residues His-79, Pro-99, and Ala-882 are highly conserved in all analyzed species (supplemental Fig. S1*A*), indicating a potentially important functional role. To experimentally assess the effects of SNP-related amino acid changes, we generated TLR9 expression constructs harboring point mutations corresponding to the eight nonsynonymous TLR9 variants reported in SNP databases (which are henceforth referred to as "TLR9 mutants"). We first assessed their ability to respond to the TLR9 ligand CpG-ODN 2006 in a Dual-Luciferase assay in HEK293 cells. As evident from Fig. 1*B*, most mutants signal comparably with WT in response to CpG-ODN. Interestingly, M400I and P99L consistently showed reduced luciferase production compared with WT TLR9. To verify comparable protein expression levels, HA-tagged, YFP-tagged, and untagged WT and mutant TLR9-transfected HEK cells were analyzed by anti-HA immunoblot (Fig. 1*C*), YFP flow cytometry, or anti-TLR9 immunoblot (data not shown), respectively. As we were interested to see whether the loss-of-function variants P99L and M400I would affect the responsiveness of WT TLR9 to CpG-ODN, we stimulated HEK293 cells transfected with a constant total amount of TLR9 plasmids (50 ng) but with an increasing molar ratio of WT to mutant TLR9 (50:0, 25:25, 10:40, and 5:45 ng) with CpG-ODN (Fig. 1*D*). In this system, M400I weakly affected signaling through WT TLR9, whereas P99L lead to attenuated NF-κB activation at higher plasmid ratios.

The TLR9 Variant P99L Is Nonresponsive to Artificial and Physiological TLR9 DNA Ligands—Having identified interesting functional phenovariants of TLR9, P99L and M400I, we sought to characterize further the strongest phenovariant, P99L. To assess whether the loss-of-function effects observed for NF-κB-driven luciferase production also pertained to the level of cytokine induction at the mRNA level, total RNA extracts from WT, or mutant TLR9-transfected HEK293 cells with or without stimulation with CpG-ODN were prepared and analyzed by real-time PCR for the induction of IL-8, TNF-α (Fig. 2, *A* and *B*), CCL5, and IFN-α and -β (data not shown). We found that in the HEK293 system, WT TLR9 stimulation did not induce CCL5, IFN-α or -β but led to a weak induction of IL-8 and TNF-α mRNA transcription relative to the housekeeping gene hypoxanthine phosphoribosyltransferase-1. P99L stimulation, on the other hand, induced neither IL-8, TNF-α, nor the remaining other cytokines, confirming the loss-of-function effect observed in NF-κB reporter assays on the transcriptional level. We next sought to assess whether loss in signaling could be rescued by higher concentration of the ligand and conducted CpG-ODN 2006 titrations from 1 to 16 μM in TLR9-transfected HEK293 cells. Fig. 2*C* shows that for WT TLR9 the level of activation did not increase at concentrations higher than 1 μM CpG-ODN 2006. On the other hand, for P99L even at high CpG-ODN concentrations, NF-κB-driven luciferase production did not increase significantly above background, suggesting that P99L is strongly hyporesponsive to CpG-ODN 2006. There are several classes of CpG-ODN with distinct properties regarding their three-dimensional structure, uptake, and intracellular trafficking (5, 38). We therefore wondered whether the hyporesponsiveness of P99L extended from the B-class ODN 2006 to the C-class ODN M362, which physiologically combines the properties of A- and B-class ODN (5). As evident from Fig. 2*D*, WT TLR9 stimulation with M362 lead to a dose-dependent increase in NF-κB-driven luciferase production, whereas M362 did not elicit any response above background via P99L even at high (8 μM) concentrations. Nei-

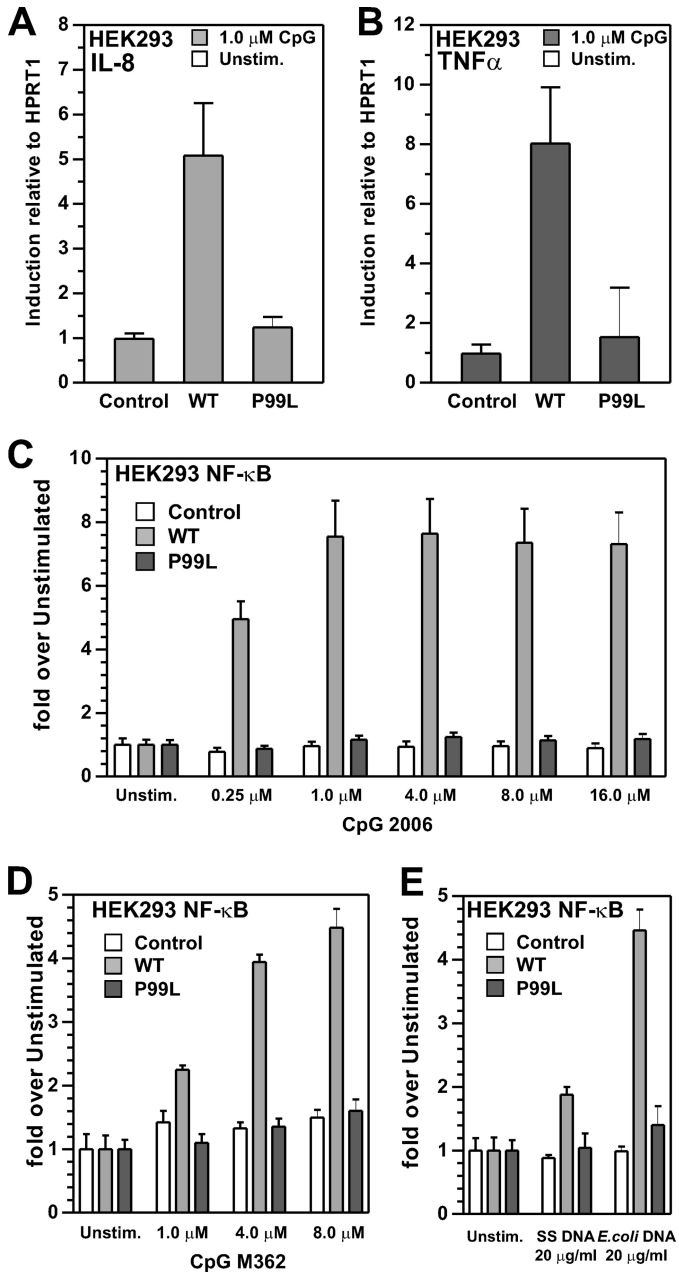

FIGURE 2. **Functional impairment of P99L pertains to the induction of different cytokines and different TLR9 ligands.** P99L fails to induce IL-8 (*A*) and TNF-α (*B*) mRNA. HEK293 cells were transfected with WT TLR9 or P99L and stimulated with CpG-ODN 2006, and total RNA was extracted. Upon preparation of cDNA, LightCycler real-time PCR was performed using universal probes and primers for specific cytokine and control genes (hypoxanthine phosphoribosyltransferase-1). One of two representative experiments is shown (technical triplicates + S.D.). *C* and *D*, higher ligand concentrations do not rescue responsiveness of P99L. HEK293 cells transfected with WT TLR9 or P99L were stimulated with different concentrations (as indicated) of CpG-ODN 2006 (B-class) (*C*) or M362 (C-class) (*D*), and NF-κB activation was measured by Dual-Luciferase assay. One of two representative experiments is shown (triplicates + S.D.). *E*, cells were transfected as above and treated with DOTAP-complexed *E. coli* and salmon sperm DNA. NF-κB activation was measured by Dual-Luciferase assay TLR9 mutants express to similar levels as WT. One of two representative experiments is shown (triplicates + S.D.). *Unstim.*, unstimulated.

ther WT nor P99L responded to the A-class ODN 2216 (data not shown). We finally stimulated TLR9-transfected HEK293 cells with bacterial DNA, the physiological ligand of TLR9 (27). Fig. 2*E* shows that WT TLR9-transfected HEK293 cells showed a marked response to *Escherichia coli* but not non-salmon sperm control DNA. P99L-transfected HEK293 cells, on the other hand, were nonresponsive to any kind of transfected DNA. Taken together, these data imply that the TLR9 variant P99L has lost the ability to respond to any type of artificial or naturally occurring DNA ligands.

Proline 99 Is Involved in CpG-ODN Sensing but Not Binding—We were intrigued to conduct a preliminary analysis into the molecular cause for the observed loss of function of TLR9 P99L. Although P99L maps to the TLR9 ectodomain and its dominant negative effect suggested localization into the same subcellular compartment as WT TLR9, we sought to verify by confocal immunofluorescence microscopy whether mislocalization could be the cause for the observed loss-of-function effect. In resting cells, TLR9 is resident in the endoplasmic reticulum, and its transmembrane region is required for trafficking to endosomes, which is orchestrated by the protein Unc93B (39, 40). Co-localization of WT and P99L was confirmed using YPet-tagged WT TLR9 and HA-tagged P99L in transfected HEK293 cells, and there was no difference in the relative localization of TLR9 WT or P99L with the endoplasmic reticulum protein calreticulin (supplemental Fig. S2). To next investigate whether the formation of pre-existing dimers shown earlier (41) was compromised by the P99L mutation, we performed anti-HA co-immunoprecipitations from HEK293 cells transfected with YFP-tagged WT TLR9 and HA-tagged TLR9 mutants. As shown in Fig. 3*A*, TLR9 P99L-HA was co-immunoprecipitated by WT TLR9-YFP to a similar extent as WT TLR9-HA, suggesting that dimer formation was not altered by the P99L mutation. We therefore concluded that the defect in signaling observed for P99L was most likely due to abrogated ligand binding or recognition. Using 3′-biotinylated CpG-ODN 2006 (confirmed to be active in signaling assays, data not shown), we performed an established pulldown assay to investigate CpG-ODN by P99L (10, 41). A mutation shown to decrease CpG-ODN recognition and binding in murine TLR9, R74E, was generated in human TLR9 and included as a negative control (10). R74E mutation also decreases responsiveness to CpG-ODN 2006 in human TLR9 (supplemental Fig. S3). As shown in Fig. 3, *B* and C, R74E, like its murine counterpart, was only pulled down to 32% of WT TLR9. This was not due to impaired dimer formation as shown in Fig. 3*A*, which was comparable with WT and P99L. We were surprised to find that although signaling was completely impaired, P99L-HA was repeatedly pulled down by the biotinylated ODN to ~75% compared with WT TLR9-HA. To visualize the correlation between signaling and binding, we divided the percentage of signaling by the percentage of binding of the P99L and R74E mutants in comparison to WT TLR9 (see "Experimental Procedures"). Fig. 3*D* clearly illustrates that for R74E the reduction in signaling correlates with a reduction of binding. For P99L, on the other hand, signaling is impaired more severely than could be suspected from the reduction in binding. These data suggest that TLR9 P99L is able to bind CpG-ODN but unable to respond to its ligand.

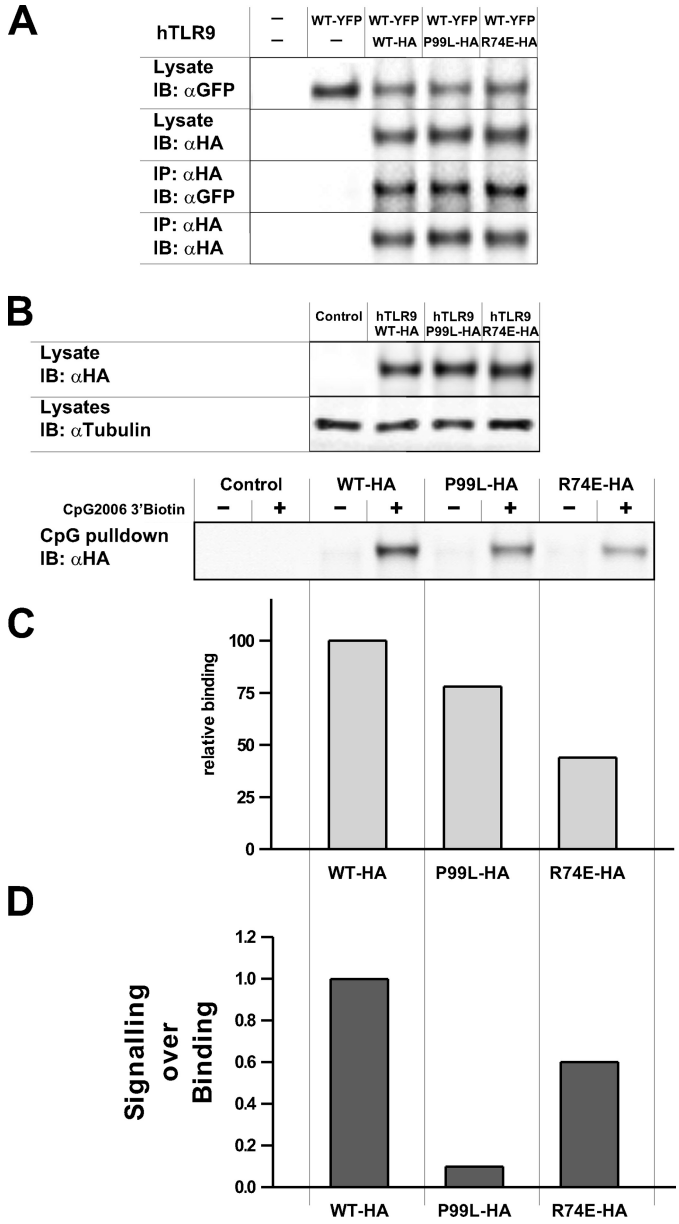

FIGURE 3. **P99L is involved in CpG-ODN sensing but not binding.** *A*, P99L maintains dimer formation. HEK293 cells were co-transfected with WT TLR9-YFP and HA-tagged WT TLR9 or P99L and lysed, and complexes were isolated by anti-HA coimmunoprecipitation. Complexes and untreated lysates were analyzed by anti-HA, anti-GFP immunoblot. One of two representative experiments is shown. *B* and *C*, P99L is precipitated by a biotinylated CpG-ODN. HEK293 cells transfected with HA-tagged WT TLR9 or P99L were lysed and incubated with biotinylated CpG-ODN 2006, and complexes were isolated using streptavidin-agarose and analyzed by anti-HA immunoblot (*IB*; *B*), and band intensities were quantified (relative to WT in % as described under "Experimental Procedures," *C*). One of two representative experiments is shown. *D*, correlation between signaling and binding properties of P99L and R74E relative to WT TLR9. Relative properties were divided and plotted.

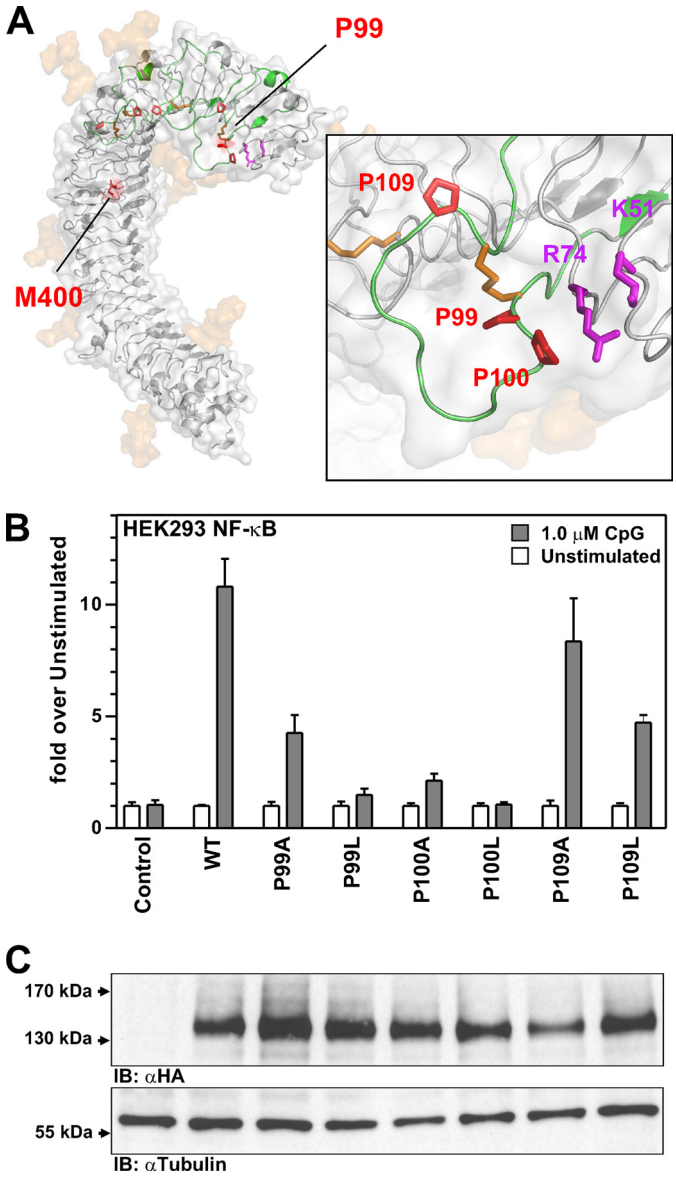

FIGURE 4. **Pro-99 is one of several LRR2 insert prolines required for CpG-ODN recognition.** *A*, model of the TLR9 ECD with identified functional SNPs P99L and M400I highlighted. Proline-rich LRR2, -5, and -8 are shown in *green*, and putative *N*-glycans are shown in *orange*. Binding site 1 residues (Arg-74 and Lys-51) are shown in *magenta*. Close-up view shows an LRR2 insertion harboring Pro-99. *B*, mutations in several LRR2 prolines compromise receptor function but not expression levels. HEK293 cells transfected with HA-tagged WT TLR9 and different TLR9 mutants were analyzed as before by Dual-Luciferase assay. One of three representative experiments is shown (triplicates + S.D.). *C*, expression levels of different TLR9 proline mutants. HEK293 cells transfected as above were lysed and analyzed by anti-HA immunoblot. One of three representative experiments is shown.

Pro-99 Is One of Several Proline Residues in the LRR2 Insertion Required for Proper CpG-ODN Recognition by TLR9—To address the question of ligand recognition in a structural framework, we generated a homology model of the human TLR9 ECD and for the P99L variant (29). As Fig. 4*A* shows, P99L maps to the loop insertion in the irregular LRR2 shown earlier to be required for full CpG-ODN signaling in murine TLR9 (10). According to our models both surface charge, potentially an important determinant for nucleic acid binding, and hydrophobicity were not significantly altered (data not shown). Interestingly, LRR2 and other insertions in TLR9 are rich in conserved proline residues (Fig. 4*A* and supplemental Fig. S4). We wondered whether Pro-99 could fulfill a particular sterical role in CpG sensing, in keeping with its high degree of conservation and the fact that prolines are known to maintain rigid backbone conformations. To probe what other amino acids may be tolerated at position 99 and at other positions in this loop, we generated TLR9 P99A, P100A, P100L, P109A, and P109L mutants

and investigated their ability to respond to CpG-ODN. Most of these mutants showed reduced responsiveness to CpG-ODN with regard to NF-κB activation (Fig. 4*B*) but expressed similarly to WT (as shown in Fig. 4*C* for HA-tagged constructs). In general, alanine mutations were not as deleterious as mutations to the bulkier leucine, but full signaling could not be rescued even at very high ligand concentrations (16 μM) as determined for P99A (supplemental Fig. S5). Taken together, this suggests that Pro-99 and other prolines in LRR insertions play a particular role in CpG-ODN sensing and that mutation to leucine as occurring in the SNP variant P99L disturbs this recognition region in TLR9.

P99L Is a Rare Sequence Variant of Human TLR9—Our functional analyses in a cellular model system imply that the SNP variant corresponding to P99L, rs5743844, might have epidemiological relevance, although little is known about its frequency in the population. Within the HapMap project, one heterozygous individual has been identified among 113 Europeans (HapMap CEU population; Utah residents with Northern and Western European ancestry; (37)), but none in a small number of African Americans or Asians according to the NCBI dbSNP database and HapMap. To gain a deeper insight into the prevalence of P99L, we investigated its presence healthy Caucasian blood donors ($n = 235$), and in case control study collectives for several infectious diseases from different ethnic groups, namely invasive pneumococcal disease from the UK (222 cases, 282 controls), bacteraemia from Kenya (149 cases, 220 controls), and leprosy from India (227 cases, 166 controls). The SNP for P99L was not found to be polymorphic in healthy donors or any of these sample collections. On the other hand, the P99L genotype was verified in the heterozygous P99L CEU individual and in two of her eight children by restriction fragment length polymorphism digest assay (data not shown). Proliferation experiments in Epstein-Barr virus-immortalized B cells of these individuals proved inconclusive due to the high level of CpG-independent basal activation, which may be a result of viral transformation (42). The M400I (rs41308230) polymorphism was also genotyped in these sample collections and confirmed to be extremely rare (monomorphic in Kenya and India) but naturally occurring in the UK population: rs41308230 was found to be heterozygous in one invasive pneumococcal disease case and one control. In conclusion, our data suggest that the P99L and M400I SNPs are very rare TLR9 variants with an expected frequency of <1% in the ethnic groups investigated thus far.

DISCUSSION

The aim of this study was to investigate whether reported nonsynonymous SNP variants of TLR9 would be associated with altered function of this innate immune receptor. Additionally, we were interested to use reported variants as probes to study TLR9 function on the molecular level. We addressed these questions by characterizing several point mutants of WT TLR9 corresponding to naturally occurring variants in HEK293 cells. Of eight mutants investigated here, we found that two amino acid alterations modulated receptor activity profoundly, P99L and M400I. Interestingly, the two functional variants exclusively mapped to the receptor ECD, and none of the TIR domain variants (R863Q, A882T) had any effect on signaling, despite the fact that TIR domains are highly conserved. On the other hand, Arg-863 is replaced with the less charged glutamine in several primates, and the change of A882T may be too conservative to alter intracellular signaling (*cf.* supplemental Fig. S1*A*). Additional mutations of these residues may help to explore their functional role, thereby defining the structural principles of TLR9 TIR domain interactions which are currently unknown.

Generating a three-dimensional model of the human TLR9 ectodomain and analyzing sequence conservation allowed us to interpret the obtained functional results for ECD variants. For the ECD variants that signaled normally (R5C, M58R, H79Q, R546Q), sequence conservation was generally low, suggesting these substitutions may be well tolerated (*cf.* supplemental Fig. S1). For H79Q, we had expected a loss-of-function effect due to its proximity to R74E in the proposed binding site 1 in murine TLR9 (10) and due to its high degree of conservation (*cf.* supplemental Fig. S1*A*). On the other hand, mutation of His-79 to glutamate in murine TLR9 maintained proper receptor function (10), suggesting that His-79 is of limited importance for signaling in both human and murine TLR9. Among the ECD variants that did affect receptor activity, M400I is located on the molecular surface in LRR13 and, according to our model, is located on the glycan-free B-side of the receptor, between the two reported binding sites for CpG-ODN (site 1: Lys-51, Arg-74 (10); site 2: Asp-535 and Tyr-537 (43)). It is thus conceivable that M400I is contributing to CpG-ODN sensing, albeit to a limited extent as illustrated by only a subtle attenuation of NF-κB activation (~40% of WT). The methionine at position 400 is conserved in several mammals, but leucine is also frequently found (supplemental Fig. S1*A*). Thus, the change to isoleucine is rather conservative and may explain the residual level of receptor responsiveness. P99L is located in the LRR2 loop insertion, a region essentially required for CpG-ODN recognition in murine TLR9. The strong conservation of this (and adjacent) proline in all mammals (*cf.* supplemental Figs. S1*A* and S4) hints to a particular role of this residue for TLR9 function. We conducted a series of experiments to obtain an idea of the underlying mechanism. Expression and confocal microscopy studies suggest that the loss-of-function effect of P99L was not due to aberrant expression or localization, respectively, as TLR9 WT and P99L receptors expressed to similar levels and co-localized in the endoplasmic reticulum of resting cells. That localization upon CpG uptake and recognition is unaltered was also indirectly confirmed by the observed dominant-negative effect, which would strictly require that WT and mutants are found in the same subcellular compartment during signal generation by WT TLR9. Our modeling studies rather suggest that Pro-99 may fulfill a particular structural role in the LRR2 loop connected to CpG-ODN sensing. In fact, perturbation of the loop region at position 99 by a bulky amino acid such as leucine renders the receptor dysfunctional, whereas alanine can be partially accommodated (*cf.* Fig. 4). The importance of proline and cysteine residues for optimal TLR9 CpG responsiveness was recently demonstrated and may be connected to their imparted sterical rigidity (44), potentially a requirement for relaying conformational changes across the

ECD and toward the TIR domain. According to our CpG-ODN pulldown data, P99L binds CpG-ODN with similar affinity compared with WT. This would imply that the P99L mutation compromises the ability of TLR9 to respond but not bind to its ligand. In fact, in CpG-ODN titration experiments, even high CpG-ODN concentrations are unable to rescue P99L-mediated NF-κB activation (*cf.* Fig. 2*C*). The data presented here fit a model of TLR9 activation (10, 44) that requires binding of CpG-ODN by the binding patches Lys-51/Arg-74 and Asp-535/Tyr-537 and contributions by LRR insertions for full receptor responsiveness. Additional mutagenesis or binding studies outside the scope of this present manuscript may more fully unravel the contribution of Pro-99 and other LRR2 insert residues on CpG-ODN detection. Our current data do not lend support to the recent notion of a C-terminal cleaved fragment of the TLR9 ECD representing the actual CpG-ODN sensor but rather point to a distinct role of the TLR9 N terminus in CpG-ODN sensing as proposed earlier (10). TLR9 cleavage may nevertheless represent a regulatory step upon sensing of the CpG motif in the context of the full-length receptor. Additional structural and biochemical studies are required to further verify the impact of ECD cleavage and this model of CpG-ODN recognition in general. Identification of individual critical residues for this process such as Pro-99 may serve as a starting point, and additional structure-guided approaches may also allow the rational improvement of currently available therapeutic CpG-ODN.

We were very much interested in the frequencies of the SNPs corresponding to function-affecting variants as carrying these rare alleles of TLR9 might render individuals more or less susceptible to bacterial or viral infections as illustrated by the phenotype of *Tlr9*-deficient mice (see Ref. 45 and references therein). Additionally, TLR9 SNPs may influence an individual's responsiveness to CpG-ODN therapies that are being evaluated in clinical trials (5). On the whole, little is known about the effect of TLR9 variants in humans thus far (18). For example, although *TLR9* falls into an systemic lupus erythematosus susceptibility region (see Refs. 6 and 46 and the introduction), several studies omitted the SNPs characterized here probably due to their low anticipated frequencies (24, 25, 47). In the context of infectious disease, an association between TLR9 SNPs with HIV (21, 22) but not malaria (26) was observed, but unfortunately neither P99L nor M400I were included in this analysis either.

The scarcity of epidemiological information on P99L and M400I therefore prompted us to determine the frequency of these functional TLR9 variants in healthy controls and patients for infectious diseases. We identified two heterozygous carriers of rs41308230 (M400I) in our UK study group (2/369), confirming the existence of this polymorphism in Caucasians. Its frequency and epidemiological significance cannot be fully assessed at this stage, but our functional data warrant the inclusion of this SNP in future studies. P99L was first described in 1/20 North Americans of undefined ethnic origin (19) and subsequently confirmed in the Utah residents of European origin (CEU) HapMap group (1/112; Ref. 37). We confirmed P99L in this individual and two of her eight children. We were surprised to find no heterozygous carriers for P99L in sample sizes of up to 400 individuals and from different ethnic backgrounds (see "Experimental Procedures"). This suggests that this variant can be considered relatively rare (< 1%) in the European, African, and Indian populations.

Examples for rare sequence variants in other components of the innate immune system such as RIG-I (48), TLR3 (14), or Mal (MyD88 adaptor-like) (49) may reflect the strong negative selective pressures encountered by these variants during human evolution. Likewise, our experimental data suggest that this mutation is functional, and the negative selective pressure may explain its rarity across different populations. It is however conceivable that under certain circumstances these variants may offer a selective advantage as proposed in the case of heterozygous carriage of Mal S180L (50), where attenuated cytokine production upon infection was put forward as potentially beneficial to the host. Our data on the combination of WT TLR9 with P99L or M400I, as in the case of heterozygous carriage of rs5743844 or rs41308230, do in fact imply that cytokine induction may be attenuated (*cf.* Fig. 1*C*). The finding that P99L was affected in its responsiveness to the physiological ligand of TLR9, bacterial DNA, and two different classes of CpG-ODN (*cf.* Fig. 2) suggests that immune responses upon microbial encounter or therapeutic intervention (5) may be modulated in P99L carriers. Comparing the effects of CpG stimulation on the proliferation of WT and P99L heterozygous immortalized B cells was an attempt to investigate the physiological significance of P99L (data not shown). As immortalization appears to lead to high levels of CpG-independent basal activation, it would probably be more informative to investigate immunological outcomes in plasmacytoid dendritic cells or B cells freshly isolated from blood samples of heterozygous carriers of M400I or P99L upon exposure to microbial pathogens or CpG-ODN. Due to their low frequencies, this may be difficult to accomplish. At this stage, it is intriguing to speculate whether poor or non-responders in ongoing clinical trials may be carriers for these hyporesponsive TLR9 variants. Although more frequent variants may have a wider yet more subtle effect on disease susceptibility in the population at large, rare variants such as P99L or M400I may contribute drastically to susceptibility on an individual level (15, 51, 52). For this reason, larger epidemiological studies including these rare but functional SNPs are warranted. Additionally, the identification and functional characterization of additional TLR9 variants may prove fruitful in an epidemiological context and serve as the means to characterize the function of this pivotal TLR further.

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