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The common R71H-G230A-R293Q (*HAQ*) human *TMEM173* is a null allele

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Abstract

TMEM173 encodes MPYS/STING, is an innate immune sensor for cyclic dinucleotides (CDNs) playing a critical role in infection, inflammation, and cancer. The R71H-G230A-R293Q (*HAQ*) of *TMEM173* is the second most common human *TMEM173* allele. Here, using data from 1000Genome Project, we found that homozygous *HAQ* individuals account for ~16.1% of East Asians and ~2.8% of Europeans while Africans have no homozygous *HAQ* individuals. Using B cells from homozygous *HAQ* carriers, we found, surprisingly, that *HAQ/HAQ* carriers express extremely low MPYS protein and have decreased *TMEM173* transcript. Consequently, the *HAQ/HAQ* B cells do not respond to CDNs. We subsequently generated an *HAQ* knock-in mouse expressing mouse-equivalent of the *HAQ* allele (mHAQ). The mHAQ mouse has decreased MPYS protein in B cells, T cells, Ly6C^{hi} monocytes, bone-marrow-derived DC and lung tissue. The mHAQ mouse also does not respond to CDNs in vitro and in vivo. Lastly, Pneumovax®23 whose efficacy depends on *TMEM173*, is less effective in the mHAQ mice than the WT mice. We conclude that *HAQ* is a null *TMEM173* allele. Our findings have a significant impact on research related to MPYS-mediated human diseases and medicine.

Introduction

Early detection of invasive pathogens is achieved by germline encoded innate immune sensors. *TMEM173* encodes an endoplasmic reticulum (ER) associated molecule MPYS (also known as MITA and STING)(1–3). MPYS is a cytosolic sensor for cyclic dinucleotides (CDNs) including bacterial CDNs, cyclic di-AMP (CDA), cyclic di-GMP (CDG), and mammalian CDN 2′5′-3′5′-cyclic GMP-AMP (2′3′-cGAMP) generated during cytosolic DNA sensing(4–6). Consequently, MPYS is critical for host defense against DNA

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viruses(7), RNA viruses(7, 8), intracellular bacteria(9, 10) and extracellular bacteria(11, 12) in mice. MPYS also plays a key role in the development of auto-inflammatory diseases in mice(13–15) and STING-associated vasculopathy with onset in infancy (SAVI) in humans(16, 17). Last, there are on-going efforts to develop MPYS/STING-targeting immunotherapy for cancer and infectious diseases(11, 18–22).

We first showed that human *TMEM173* gene has significant heterogeneity (23). We identified *R232* of *TMEM173*, not *H232*, as the most prevalent allele (*wt*) in the human population(23). However, we found that only ~50% of Americans are *R232/R232*(23). We further identified *HAQ*, which contains three non-synonymous SNPs, R71H-G230A-R293Q, as the second most common human *TMEM173* allele and estimated that ~3% of Americans are homozygous for *HAQ*(23). Transiently overexpressing *HAQ* in 293T cells leads to >90% decrease of type I IFN production, the hallmark function of MPYS/STING(23). 293T cells stably transfected with *HAQ* also have decreased response to CDN stimulation (6, 24). Here, we studied the endogenous function of the *HAQ* allele using human cells from homozygous *HAQ* carriers and the mHAQ knock-in mouse. We discovered, unexpectedly, that the *HAQ TMEM173* has decreased protein expression (~90%) and did not respond to CDN stimulation in vivo and in vitro.

Materials and Methods

Generation of an *HAQ*-MPYS knock-in mice

The linearized targeting vector (Figure S3A), which covers ~10 kb of the genomic region in *MPYS* locus on mouse chromosome 18, was transfected into JM8A3.N1 embryonic stem (ES) cells originated from the C57BL/6 strain, followed by the selection for neomycin positive and diphtheria toxin (DTA) negative clones. Targeted clones were screened by PCR. Positive ES clone was subjected to the generation of chimera mice by injection using C57BL/6J blastocysts as the host. The male chimeras (chimerism >95% determined by coat color) were mated with C57BL/6J female mice for germline transmission. Successful germline transmission was confirmed by PCR-sequencing (Figure S3B). The heterozygous mice were bred to Actin-flpase mice (The Jackson Laboratory, B6.Cg-Tg(ACTFLPe)9205Dym/J) (Figure S3A) to remove the neo gene and make the *HAQ*-MPYS knock-in mouse. Animals were generated at the National Jewish Health Mouse Genetics Core Facility. Animal care and handling was performed according to institutional animal care and use committee guidelines.

Mice

Six- to twelve-week-old mice, both males and females, were used for all experiments. MPYS$^{-/-}$ mice (Tmem173$^{<tm1Camb>}$) were described previously(25). All mice were on a C57BL/6 background. Mice were housed and bred in the Animal Research Facility at Albany Medical College and the University of Florida. All experiments with mice were performed by the regulations and approval of the Institutional Animal Care and Use Committee from Albany Medical College or the University of Florida.

Reagent

The following reagent was obtained through BEI Resources, NIAID, NIH: Streptococcus pneumonia Family 1, Clade 2 Pneumococcal Surface Protein A (PspA UAB055) with C-Terminal Histidine Tag, Recombinant from Escherichia coli, NR-33178.

Data Mining

Human *TMEM173* genotype information was obtained from 1000Genome Project (Phase III, http://browser.1000genomes.org/index.html). Human B cells with the corresponding *TMEM173* genotypes were obtained from Coriell Cell Repositories (https://catalog.coriell.org/) and cultured in RPMI 1640 with 15% FCS, 2mM L-glutamine, 37°C under 5% CO2. Information related to *TMEM173* gene expression was obtained from The Genotype-Tissue Expression (GTEx) project (http://www.gtexportal.org/home/).

Human B cells Activation by CDNs

For CDA (Invivogen, cat#vac-cda), CDG(Invivogen, cat#vac-cdg) and 2′3′-cGAMP (Invivogen, cat#vac-cga23) activation, human B cells were harvested and suspended (5×10^6 cells/ml) in transporter buffer (26) (110mM potassium acetate, 5mM sodium acetate, 2mM magnesium acetate, 1mM EGTA, 2mM DTT, 20mM HEPE pH 7.3 and protease-inhibitor cocktail (Biotool, cat# B14011)) with 10μg/ml digitonin (Calbiochem®, cat# 300410) in the presence or absence of CDNs (10μg/ml). Cells were cultured at 37°C for 10min in 24 well plate. Afterward, cells were harvested and resuspended in the human B cell culture medium at 5×10^6 cells/ml, and cultured with or without CDNs (10μg/ml) for 5hrs. Human IFNβ was measured in cell supernatant by ELISA (PBL Bioscience, cat#41415).

To measure IRF3 nuclear translocation, cells were harvested at the end of 5hr incubation. Nuclear fraction was isolated as previous reported (27) and run on a 10% Mini-PROTEAN® TGX gel (BioRAD, CAT#456-1035). Abs used for western blot are IRF3 Ab (CellSignaling, cat# 43025), α-rabbit IgG-HRP (CellSignaling, cat#7074s), α-mouse IgG-HRP (CellSignaling, cat#7076s), cyclophilinB Ab (CellSignaling, cat#76952s) and Tubulin Ab (Rockland, cat#200301-880) and rabbit anti-MPYS polyclonal Ab (3).

To measure IRF3 phosphorylation, cells were harvested at the end of 5hr incubation and lysed in the RIPA buffer as previous reported (27) and run on a 10% Mini-PROTEAN® TGX gel (BioRAD, CAT#456-1035) and probed for anti-p-IRF3 (s396) (CellSignaling, cat# 4D4G).

For RpRp-ssCDA (Biolog, cat#c118-001) activation, cells were suspended in human B cell medium at 5×10^6 cells/ml. 5μg/ml RpRp-ssCDA was added directly into a medium for 5hrs. Afterward, IRF3 activation was examined as above.

Q-PCR to Determine *TMEM173* mRNA in Human B cells

Human B cells (1.2×10^6) were harvested and lysed in 350μl of RLT sample buffer with 40μM dithiothreitol. Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen, cat#74134) and reverse-transcribed using the high capacity reverse transcription kit (Applied Biosystems). Quantitative PCR (Q-PCR) was carried out on a StepOnePlusTM instrument

(Applied Biosystems) using the following primers and probes: human αActin (Fwd:5′-TCACCCACACTGTGCCCATCTACG-3′,Rev:5′-CAGCGGAACCGCTCATT GCCAATG-3′) and SYBER-Green human *TMEM173* (BioRad, cat# 10025636, Assay ID: qHsaCID0010565). Gene expression was normalized to Actin expression and relative expression of the respective gene in untreated cells.

Semi-Quantitative PCR to Amplify Full-length Human *TMEM173* gene

Total RNA was extracted from human B cells using the RNeasy Plus Mini kit (Qiagen, cat#74134). Total cDNA was made using the Superscript™ IV First-Strand Synthesis System (Invitrogen, #18091050). Full-length human *TMEM173* gene (1379bp) was amplified with following primers: *TMEM173-For*: 5′-TTGGCTGAGTGTGTGGAGTC-3′; *TMEM173-Rev*: 5′-CAGTCCAGAGGCTTGGAGAC-3′. Human *GAPDH* primers (R&D Systems, # RDP39) were used to amplify the *GAPDH* cDNA as a control.

BMDM and BMDC Activation

Bone marrow cells were cultured in RPMI (Invitrogen, cat#11965118) with 10% FBS, 2mM L-glutamine, 1mM Sodium pyruvate, 10mM HEPES buffer, 1% Non-essential amino acids, 50μM 2-Mercaptoethanol, 1% Pen/Strep, 20ng/ml GM-CSF (Kingfisher, cat# RP0407M) or 20ng/ml M-CSF (Kingfisher, cat# RP0462M). The medium was changed at day 3 and 6. At day 6, cells (1×10^6) were transferred to a 24-well plate with fresh medium. Cells were activated at day 7 with 10μg/ml CDA, CDG, 2′3′-cGAMP or 5μg/ml Rp-Rp-ssCDA in culture directly. Mouse IFNβ was measured in culture supernatant after 5hrs by ELISA (PBL Bioscience, cat#42410). Separately, BMDM and BMDC were activated with 5μg/ml HSV DNA (Invivogen, cat# tlrl-hsv60n) and Vaccinia virus DNA (Invivogen, cat# tlrl-vav70n) transfected with lipofectamine®2000(27) and mouse IFNβ was measured in culture supernatant after 5hrs by ELISA. Alternatively, BMDC were activated with Heat kill streptococcus pneumonia (HKSP) (10^8c.f.u/ml) (Invivogen, cat# tlrl-hksp), LPS from Salmonella (25ng/ml) (Sigma, cat# L7261), Imiquimod (4ng/ml) (Invivogen, cat# tlrl-imqs) or CpG-ODN2395 (8ng/ml) (Invivogen, cat# tlrl-2395). Mouse TNFα and IFNβ were measured in culture supernatant after 5hrs by ELISA.

In vivo CDN Activation

Mice were intranasally administered 5μg 2′3′-cGAMP (Invivogen, cat#vac-cga23), then sacrificed after 5hrs by CO2 asphyxiation(11). Lungs were perfused with cold PBS. The harvested lungs were washed with PBS once, then stored in 0.7ml Tissue protein extraction reagent (T-PER) (Thermo Scientific, cat#78510) containing protease inhibitors (Roche, cat#11836153001) at −80°C. Later, the lung was thawed on ice and homogenized with Minilys® (Precellys, 5,000 RPM for 30sec) using Precellys lysing kit (Precellys, cat# KT03961). Lung homogenates were transferred to a 1.5ml tube and spun at 14,000g for 30min at 4°C. The supernatant was collected and analyzed for cytokine production.

Cytokine concentrations were measured by ELISA kits from eBioscience. The ELISA kits used were IL-5 (cat#88-7054), IL-12/p70 (cat#88-7921), IL-13 (cat#88-7137), IL-17A (cat#88-7371), TNF-α (cat#88-7324), IFN-λ (cat#88-7284), and IFN-γ (cat#88-7314).

Intranasal CDN Immunization

Groups of mice (4 per group) were intranasally vaccinated with 5μg 2′3′-cGAMP adjuvanted PspA (2μg, BEI Resources) or PspA alone(11). Mice were immunized twice at 14 days interval. For intranasal vaccination, animals were anesthetized using isoflurane in an E–Z Anesthesia system (Euthanex Corp, Palmer, PA). PspA, with or without 2′3-cGAMP was administered in 20μl saline. Sera, BALF, and nasal washes were collected 14 days after the last immunization. The PspA-specific Abs were determined by ELISA. Secondary Abs used were anti-mouse IgG1-HRP (Southern Biotech, cat#1070-05), anti-mouse IgG2C-HRP (Southern Biotech, cat#1079-05), and anti-mouse IgA-HRP (Southern Biotech, cat#1040-05). To determine Ag-specific Th response, splenocytes from PspA or 2′3-cGAMP + PspA immunized mice were stimulated with 5μg/ml PspA for four days in culture. Th1, Th2, and Th17 cytokines were measured in the supernatant by ELISA.

Pneumovax®23 Immunization

A group of mice (four mice per group for the Ab experiment and ten mice per group for the survival experiment) was intramuscularly administered with 0.125μg of Pneumovax® 23 (Merck, cat#7002681601) in 50μl Ultrapure PBS (Amresco, cat#K812) or PBS alone. Blood was collected, before and after immunization at indicated time. Anti-PPS2 and PPS3 IgM were determined by ELISA. Following reagents were used Pneumococcal polysaccharide Type 3 (PPS3) (ATCC® 31-X, ID # 61810463), Pneumococcal polysaccharide Type 2 (PPS2) (ATCC® 500-X,ID# 63406999), 1× ELISA assay diluent (eBioscience, REF # 00-4202-43) and goat anti-Mouse IgM HRP (cat # 1020-05, SouthernBiotech). One month after the immunization, mice were challenged (i.n) with S. pneumonia (A66.1 strain, serotype 3, ~10^6 c.f.u in 50μl PBS). Animal health was monitored for eight days.

Human PBMC Experiments

The study was approved by the Ethics Committee of the Charité University Medicine Berlin, and participants gave informed written consent. Genomic DNA from individuals was isolated and genotyped for analysis of the *HAQ* haplotype. Three individuals carrying the *HAQ* haplotype in homozygosity and 3 carrying *R232 (WT) TMEM173* were identified.

DNA from buccal swabs was extracted using a DNA mini kit (Qiagen). Genotyping was performed by PCR using fluorescence-labeled hybridization FRET probes and melting curve analysis employing the LightCyler 480TM (Roche Diagnostics). Genotyping of the tmem173 SNP R71H was carried out using the following primer and probe: F-primer (rs11554776 S) ggagtgacacacgttgg, R-primer (Rs11554776 A) gcctagctgaggagctg, probe (rs11554776 C): ctggagtgga-XI-tgtggcgcag-PH. Primer and probes for the tmem173 SNP R293Q were as follows: F-primer (rs7380824 F): accctggtaggcaatga, R-primer (rs7380824 R): gcttagtctggtcttcctcttac, sensor probe (rs7380824 C): cctcaagtgtccggcagaagagtt-FL, anchor probe (Anc rs7380824): 640-ggcctgctcaagcctatcctcccgg-PH.

Peripheral blood samples were drawn in 50 ml EDTA-coated syringes, and PBMCs were isolated by density gradient centrifugation using sterile-filtered Histopaque®-1077 (Sigma-Aldrich Chemie Gmbh). Cells were plated at a density of 1.2×10^6 cell/well in a 24-well format and stimulated with 0.4 or 2 μg/well of Rp, Rp-ssCDA.

Total RNA was isolated from PBMCs lysates using the PerfectPure RNA Cultured Cell Kit (5prime GmbH) and reverse-transcribed using the high capacity reverse transcription kit (Applied Biosystems). Quantitative PCR (Q-PCR) was carried out on an ABI 7300 instrument (Applied Biosystems) using the following primers and probes: ifnb: F-primer: ccaacaagtgtctcctccaaatt, R-primer: gtaggaatccaagcaagtgtagct, probe: FAM-tgttgtgcttctccactacagctctttcca-TAMRA. Analysis of tmen173 expression was performed with a TaqMan gene expression assay Hs00736958_m1 (Applied Biosystems). Gene expression was normalized to GAPDH expression and relative expression of the respective gene in untreated cells.

Statistical Analysis

All data are expressed as means ± SEM. Statistical significance was evaluated using Prism 5.0 software to perform a Student's t-test (unpaired, two-tailed) for comparison between mean values.

Results

Homozygous *HAQ* individuals are common in non-Africans

We previously estimated that ~3% of Americans are *HAQ*/*HAQ* (23). To expand this knowledge to other ethnic groups, we extracted *TMEM173* genotypes data from the 1000Genome Project (Phase III). Among the five ethnic groups defined in the 1000Genome Project, we found that *HAQ*/*HAQ* is most common in East Asian (~16.07%), followed by South American (~7.78%), South Asian (~6.75%) and European (~2.78%) (Table 1). Surprisingly, no homozygous *HAQ* individual is found in Africans (Table 1). Instead, ~4.39% of Africans are AQ/AQ (G230A-R293Q), which is not found in non-Africans (Table 1). We concluded that human *TMEM173* gene has not only great heterogeneity but also show significant population stratification.

Homozygous *HAQ* B cells have very low MPY protein expression compared to the *R232* B cells

To study the function of *HAQ*, we obtained EBV-transformed human B cells from homozygous *HAQ* individuals identified in the 1000Genome Project. These cells are distributed by National Human Genome Research Institute (NHGRI) Repository at Coriell Institute. They express B-cell surface markers IgM and HLA-DR (Figure S1A). Notably, these cells also express B cell activation markers CD80, CD86 and CD69 (Figure S1A). The expression level of these markers is similar between *R232* (wt) and *HAQ* B cells (Figure S1A).

We next examined MPYS expression in these cells. Surprisingly, we found that the homozygous *HAQ* B cells from different ethnic groups have very low MPYS protein (Figure 1A, 1B, 1C & 1D). No MPYS protein was detected in the cell debris (Figure S1B) excluding the possibility that *HAQ* protein somehow may be insoluble or aggregate.

We have been using this rabbit anti-MPYS Ab since we initially identify MPYS in 2008 and its specificity has been well documented by the literature (3, 9, 23, 25). Nevertheless, to

exclude the possibility that our anti-MPYS Ab may not recognize the *HAQ* MPYS, we cloned the *HAQ TMEM173* transcript from the homozygous *HAQ* human B cells and expressed it in 293T cells, which lack the endogenous MPYS expression(23). Our anti-MPYS Ab staining showed a similar expression of the *HAQ* and *R232* of MPYS in the 293T cells (Figure S1C), which indicated that our anti-MPYS Ab recognizes the *HAQ* of MPYS as good as the *R232* of MPYS. We thus concluded that the low MPYS staining in the *HAQ* B cells (Figure 1A~1D) is indeed an indication of low MPYS protein expression.

We also compared the MPYS level in these 293T transfectants with our human B cells. We found that the endogenous MPYS level in human B cells is ~50-fold lower than that of 293T transfectants (Figure S1C), which suggested that overexpressing *TMEM173* in 293T cells likely masked the expression difference between the endogenous *R232* and *HAQ* of *TMEM173*.

Homozygous *HAQ* human B cells are defective in response to natural CDNs

MPYS senses natural CDNs, including the bacterial CDN CDA, CDG and mammalian CDN 2′3′-cGAMP(4, 5, 28, 29). We hypothesized that *HAQ*/*HAQ* cells would not respond to these CDNs due to the low MPYS expression.

Directly adding CDN in the human B cells culture did not activate these cells (Figure 1E &1G, Figure S2A, S2C, S2E & 2F, no digitonin). To deliver CDN into the cytosol, human B cells were reversibly permeabilized with digitonin in the presence of 2′3′-cGAMP. Activation of MPYS by CDNs leads to phosphorylation and nuclear translocation of IRF3 and subsequently IFNβ production. In Spanish and Chinese samples, 2′3′-cGAMP activates IRF3 translocation and IFNβ production in *R232/R232* individuals but not the *HAQ*/*HAQ* B cells (Figure 1E–1H). Furthermore, 2′3′-cGAMP did not induce IRF3 phosphorylation in the *HAQ/HAQ* cells from Chinese, Spanish, British and Italian (Figure 1I). Similar observations were made in *HAQ/HAQ* samples in response to CDA and CDG (Figure S2A–2F).

CDN stimulation also activates MPYS-dependent NF-κB signaling(1, 27, 30). However, we found that these B cells have constitutively activated NF-κB as indicated by the presence of nuclear RelA and RelB (Figure S1D). CDN activation, which increases nuclear IRF3, did not further increase nuclear RelA or RelB (Figure S1D). This is consistent with our observation that these B cells have an activated phenotype (Figure S1A).

Homozygous *HAQ* human B cells are defective in response to synthetic CDN

Recently, a synthetic CDN, RpRp-ssCDA, was showed to activate all major human *TMEM173* variants overexpressed in 293T cells (18, 31). We next examined this synthetic CDN in human *HAQ/HAQ* B cells. First, we found, surprisingly, that RpRp-ssCDA can activate human B cells in medium without the need of permeabilization (Figure 2A, 2C, 2E & 2J). Second, *HAQ/HAQ* cells from British, Italian, Spanish and Chinese, are all defective in IRF3 activation and IFNβ production in response to RpRp-ssCDA (Figure 2A–2H). Lastly, PBMC from three German *HAQ/HAQ* individuals also had a defective IFNβ response to RpRp-ssCDA compared to the *R232/R232* individuals (Figure 2I). RpRp-ssCDA can induce Type I IFN production in the Goldenticket mouse, which lacks detectable MPYS/

STING protein (18), which may explain the residual IFNβ by RpRp-ssCDA in some samples. We concluded that homozygous *HAQ* B cells are defective in response to the synthetic CDN RpRp-ssCDA.

Homozygous *H232* and the *HAQ/H232* human B cells are defective in response to natural and synthetic CDNs

The H232 of MPYS has low binding affinity for CDNs (6). We found that MPYS expression in *H232/H232* human B cells is similar to the *R232/R232* while the *HAQ*/*H232* B cells have decreased MPYS expression likely due to the presence of the *HAQ* allele (Figure 1A, 1B, 1C & 1D). We next examined their CDN responses. We found that both the homozygous *H232* and the *HAQ/H232* B cells did not have IRF3 nuclear translocation and IFNβ production in response to 2′3′-cGAMP, CDA, CDG or RpRp-ssCDA (Figure 1E–1H) (Figure S2) (Figure 2). We concluded that similar to the homozygous *HAQ* B cells, homozygous *H232,* and the *HAQ*/*H232* B cells are also defective in response to CDN.

Establish an *HAQ* mouse model

Mouse and human MPYS proteins are 82% homologous (2). To understand the in vivo significance of the *HAQ* of *TMEM173*, we generated an *HAQ* knock-in mouse (mHAQ). This knock-in mouse contains mouse equivalent of the *HAQ* mutations: C71H, I229A, and R292Q (Figure S3A). The presence of these three mutations was confirmed by sequencing (Figure S3B). Similar to the human *HAQ* B cell, we found that MPYS expression is also decreased in mHAQ spleen B cells (Figure 3A), which suggested that the mHAQ mouse recapitulates the main feature of the human *HAQ*.

The establishment of the mHAQ mouse allowed us to examine *HAQ* expression and function beyond the B cells. Indeed, we found that MPYS expression is decreased in mHAQ spleen T cells (Figure 3A) and mHAQ bone marrow Ly6C^{hi} monocytes (Figure 3B). The defect is more pronounced in the mHAQ lung (Figure 3C) and mHAQ BMDC (Figure 3D) where MPYS expression is not detectable. Notably, in naïve BMDM, IFNγ differentiated M1 macrophage or IL-4 differentiated M2 macrophage, MPYS expression is similar between the WT and mHAQ mice (Figure 3E), which may indicate a macrophage-specific regulation of MPYS protein expression.

The mHAQ mouse is defective in response to CDNs in vitro and in vivo

Human *HAQ* B cells are non-responsive to CDNs (Figure 1 and S2). We next examined CDNs response in BMDC and BMDM from the mHAQ mouse. As expected, both the mHAQ BMDM and BMDC did not produce IFNβ in response to CDA, CDG, 2′3′-cGAMP or RpRp-ssCDA (Figure 4A & 4B). The mHAQ BMDM and BMDC also did not make IFNβ in response to transfected Herpes Simplex Virus DNA or Vaccinia virus DNA (Figure 3A & 3B). As a control, BMDC from mHAQ mice has similar TNFα (Figure 3F) and IFNβ (Figure 3G) production as the WT mice when stimulated with TLR2 ligand Heat-killed streptococcus pneumonia (HKSP), TLR4 ligand LPS, TLR7 ligand imiquimod, and TLR9 ligand CpG-ODN2395.

We next examined the in vivo CDN responses in the mHAQ mouse. Intranasal administration of CDN elicits rapid cytokine productions in the lung that is important for the mucosal adjuvant activity of CDNs (11). We found that intranasal administration of 2′3′-cGAMP did not elicit lung production of TNFα, IL-12p70, IFNγ or IFNλ in the mHAQ mouse (Figure 4C). We further examined the mucosal adjuvant activity of 2′3′-cGAMP in the mHAQ mouse(32). As expected, 2′3′-cGAMP did not induce Ag-specific Ab or Th response in the mHAQ mouse (Figure 4D&4E). We concluded that the mHAQ mouse does not respond to CDN in vivo and in vitro.

Pneumovax®23 is less effective in the mHAQ mouse than the WT mice

The CDNs-MPYS/STING activation in B cells is required for polysaccharide-based vaccine activity such as Pneumovax®23 (33). Since mHAQ mice do not have functional CDNs-MPYS pathway (Figure 4), we hypothesized that Pneumovax®23 would not be effective in the mHAQ mouse. Indeed, upon intramuscular Pneumovax®23 immunization, the mHAQ mice have lower anti-PPS3 IgM (Figure 5A & 5B) and anti-Pneumococcal polysaccharide Type 2 (PPS2) IgM (Figure 5C & 5D) production than the WT mice at day 14 and 21.

To examine the protective immunity of Pneumovax®23 in the mHAQ mouse, we challenged vaccinated mice with the A66.1 strain, an invasive strain of *S. pneumococcus*. Consistent with the Ab results, Pneumovax®23 protected WT mice from the A66.1 *S. pneumococcus* infection (Figure 5E) but not the mHAQ mouse (Figure 5F). We concluded that Pneumovax®23 is not effective in the mHAQ mouse.

Homozygous *HAQ* B cells have decreased *TMEM173* transcript

We next ask why the human *HAQ* allele has low MPYS protein expression. We examined the *TMEM173* mRNA level in the homozygous *HAQ* B cells. Surprisingly, we found that *HAQ* B cells from Sri Lankan Tamil, Colombian, Japanese and Italian that have low MPYS expression (Figure 6A), all have ~40% lower *TMEM173* mRNA than their *R232/R232* ethnic controls (Figure 6B). Semi-quantitative PCR also show that the full-length human *TMEM173* transcript (~1.4kb) is decreased in the homozygous *HAQ* B cells compared to their *R232* counterparts (Figure 6C). We concluded that human *HAQ* B cells had decreased *TMEM173* transcript.

We next asked if the homozygous *HAQ* individuals have decreased *TMEM173* transcript in tissues other than B cells. To answer that, we mined data from The Genotype-Tissue Expression (GTEx) database, which compiles data regarding human gene expression related to genetic variations. We focused on the SNPs that affect the *TMEM173* transcript. We found that all three *HAQ* SNPs, rs11554776(R71H) -rs78233829(G230A) -rs7380824(R293Q), are associated with decreased *TMEM173* transcript with highly significant p values as low as 10^{-19}, 10^{-22} and 10^{-23} (Table 2). Furthermore, this decreased *TMEM173* transcript in *HAQ* individuals can be found in non-B cell dominant tissues such as Artery, fibroblasts, lung, Thyroid and Esophagus (Table 2). Thus, homozygous *HAQ* individuals have decreased *TMEM173* in tissues other than B cells.

Discussion

The common human *HAQ TMEM173* allele was first identified and characterized by us in 2011(23). We characterized the *HAQ* as a loss-of-function *TMEM173* allele because it loses >90% of the ability to stimulate IFNβ production when transiently overexpressed in the 293T cells, a hallmark function of MPYS/STING(23). In 2013, Diner, E.J., *et.al.*(5) found that the THP-1 cell, a human monocytic cell line originated from a Japanese (34) have the *HAQ* of *TMEM173*. However, it is not clear if the THP-1 cells are homozygous or heterozygous for *HAQ*. Also in 2013, Yi, G., *et. al* found that 293T cells stably expressing the *HAQ* can respond to CDN, albeit weaker than the *R232* of *TMEM173* (24). Using 293T cells stable transfectants to study *HAQ* function, nevertheless, could be misleading. The reasons are (i) the level of MPYS is 50-fold higher in the 293T cells transfectants than the endogenous MPYS (Figure S1C). (ii) Human *HAQ* cells have decreased *TMEM173* transcript (Figure 6). This feature of the *HAQ* is lost when expressing the *HAQ* cDNA in the 293T cells. In the current report, we used homozygous *HAQ* human B cells from multiple ethnic groups showed that homozygous *HAQ* B cells have very low MPYS expression compared to the *R232* B cells and do not respond to CDN in vitro. Furthermore, PBMCs from 3 homozygous *HAQ* German have decreased CDN response compared to the *R232* German. Lastly, an *HAQ* knock-in mouse has decreased MPYS expression and did not respond to CDN in vitro and in vivo. Thus, *HAQ* is indeed a loss-of-function human *TMEM173* allele likely due to its extremely low protein expression.

Two other *TMEM173* genotypes, *HAQ/H232*, and *H232/H232* also did not respond to CDN (Figure 1 & 2). The underlying molecular mechanisms are likely different. The *H232/H232* B cells, unlike the *HAQ/HAQ*, have similar MPYS expression as the *R232/R232* B cells. Previous studies found that the H232 of MPYS binds CDN (K_d ~5.3μM) much poorly than the R232 of MPYS (K_d ~0.11μM) (6). Consequently, the *H232* is severely defective in response to CDN stimulation when expressed in 293T cells (6, 29). Here, we verified this observation in our homozygous *H232* human B cells (Figure 1). The *HAQ/H232* B cells have the *HAQ* allele, which contributes to its low MPYS expression (Figure 1), and the non-functional *H232* allele. Together, they lead to the unresponsiveness to CDN in the *HAQ/H232* B cells.

It is worth noting that in all, the *HAQ/HAQ*, *HAQ/H232* and *H232/H232* genotypes, consist of ~10% of Europeans and ~31% of East Asians (Table 1). This is significant because there are tremendous interests to develop MPYS/STING-targeting immunotherapies for cancers and infectious diseases (18, 19, 21, 22, 35). It will be especially challenging to develop MPYS/STING-targeting immunotherapies for the homozygous *HAQ* individuals because of their extremely low MPYS protein expression. Indeed, we showed that the licensed pneumococcal vaccines Pneumovax®23 is less effective in the mHAQ mouse than the WT mice (Figure 5). Fu, J *et.al.*, did show that the synthetic CDN RpRp-ssCDA activates PBMCs similarly in *HAQ/HAQ* and *R232/R232* donors (18). However, the PBMC was from 1 single *HAQ/HAQ* donor (18). Here, we used three homozygous German *HAQ* individuals and found they were defective in response to RpRp-ssCDA (Figure 2I). Future development of MPYS-targeting immunotherapies must adopt the concept of personalized medicine.

Surprisingly, we found that the synthetic CDN RpRp-ssCDA is membrane permeable. Natural CDNs have two phosphate groups preventing it from directly passing through the cell membrane. To activate MPYS, which is inside cells, investigators have to use transfection or membrane permeabilizing reagents to deliver CDN to the cytosol. We previously showed that, in vivo, only pinocytosis-efficient cells such as macrophage and dendritic cells, can directly take up CDG and be activated (11). The observation that RpRp-ssCDA is cell-permeable makes it a very attractive CDN to direct activate cells that are not pinocytosis-efficient such as B cells.

Last, the null phenotype of the *HAQ* allele is likely a result of the decreased *TMEM173* transcript and the amino acids changes (R71H-G230A-R293Q) in the HAQ protein (Figure 6D). The MPYS protein level is down ~60% in the mHAQ knock-in mouse. Previously, an I199N change in the mouse *TMEM173* gene leads to a complete loss of the STING/MPYS protein expression(36). Thus, amino acid changes in MPYS protein can impact its expression. MPYS expression can also be regulated at a transcriptional level. Mouse and human *TMEM173* genes have conserved STAT1 binding sites(37). Type I and II treatments increase mouse and human *TMEM173* expression via an STAT-1 dependent mechanism (37). Nevertheless, treating homozygous human *HAQ* B cells with IFNγ did not restore MPYS protein expression (data not shown). Further studies are needed to reveal the mechanisms by which human *TMEM173* expression is controlled on the transcriptional and post-transcriptional level.

In summary, we found that human *HAQ*, the second most common *TMEM173* allele, is a null allele. The mouse model of *HAQ*, the mHAQ knock-in mouse, are not protected by Pneumovax®23. Future studies need to be done to determine the impact and mechanisms by which *HAQ*, as a loss-of-function common *TMEM173* allele, influence human diseases, and medicines. Our *HAQ* knock-in mouse will be especially valuable in this endeavor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HAQ	R71H-G230A-R293Q
CDN	cyclic dinucleotide
CDG	Cyclic di-GMP
CDA	Cyclic di-AMP
2′3′-cGAMP	GMP-AMP, 2′5-3′5′-cyclic

PspA	Pneumococcal surface protein A

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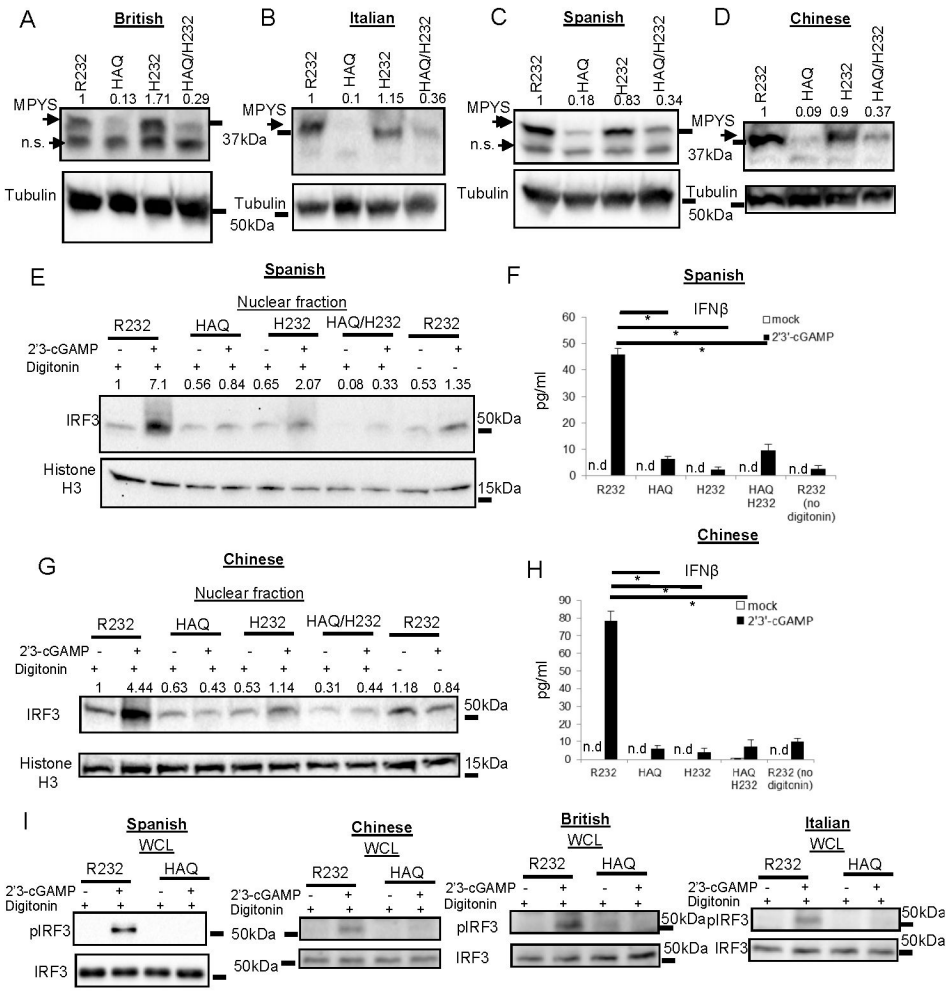

Figure 1. Homozygous *HAQ* human B cells have very low MPYS protein expression compared to the *R232* B cells and do not respond to natural CDNs

A–D. *R232/R232, HAQ/HAQ, H232/H232 and HAQ/H232* human B cells from indicated ethnic groups were lysed in RIPA buffer and probed for MPYS expression using the rabbit anti-mouse MPYS Ab (n>3). **E&G**. *R232/R232, HAQ/HAQ, H232/H232 and HAQ/H232* human B cells from indicated ethnic groups were activated with 2′3′-cGAMP (10μg/ml) for 5 h as described in Materials and Methods. Nuclear fractions were isolated. Samples were run on a SDS-PAGE gel and probed with the indicated Abs (n=3). **F&H**. Human IFNβ was measured in cell supernatant from **E&G** by ELISA (n=3). **I**. *R232/R232, HAQ/HAQ* human B cells from indicated ethnic groups were activated with 2′3′-cGAMP (10μg/ml) for 5 h as in E&G. Cells were lysed in the RIPA buffer. Whole cell lysate (WCL) were run on a SDS-PAGE gel and probed with the indicated Abs (n=2). Graph present means ± SEM from three independent experiments. The significance is represented by an asterisk, where $p<0.05$. n.s, nonspecific. n.d, not detected.

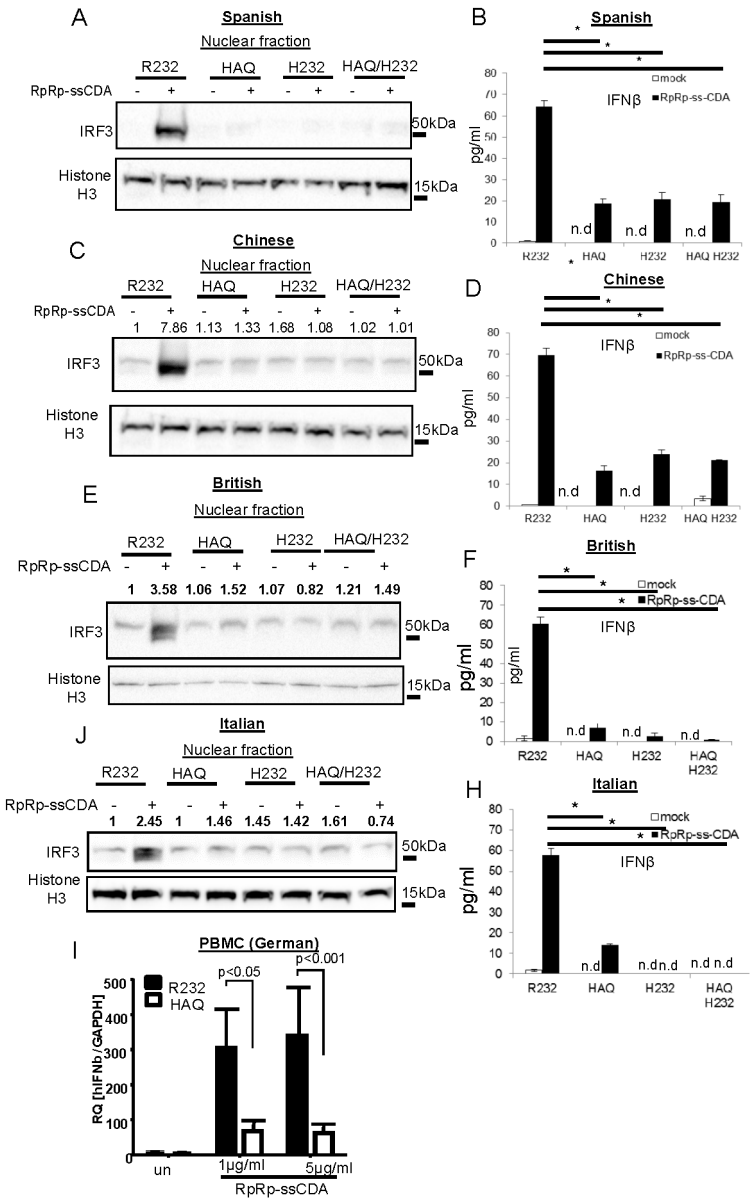

Figure 2. Homozygous *HAQ* human B cells are defective in response to synthetic CDN RpRp-ssCDA

A,C,E,J. *R232/R232, HAQ/HAQ, H232/H232 and HAQ/H232* human B cells from indicated ethnic groups were activated with RpRp-ssCDA (5μg/ml) for 5 h in culture. Nuclear fractions were isolated, run on a SDS-PAGE gel and probed with the indicated Abs (n=3). **B,D,F,H**. Human IFNβ was measured in cell supernatant from **A,C E,J** by ELISA (n=3). **I**. PBMCs from three homozygous *HAQ* and *R232* German were stimulated with RpRp-ssCDA. Relative expression of *ifnb* was determined by q-PCR (n=3). Graph present means ± SEM from three independent experiments. The significance is represented by an asterisk, where $p<0.05$. n.d, not detected.

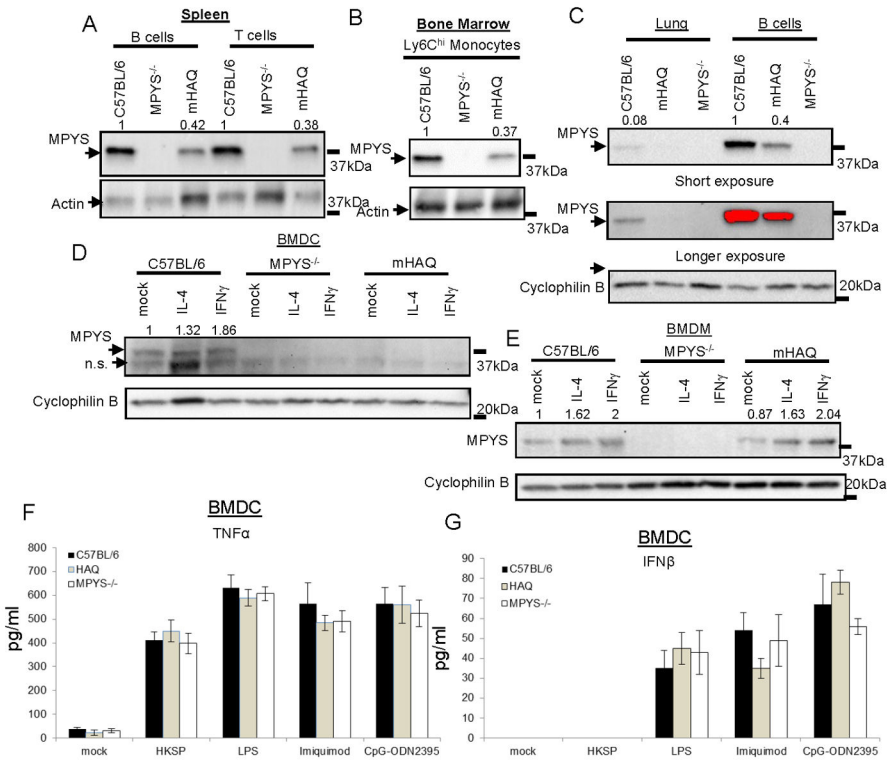

Figure 3. The *HAQ* knock-in mouse (mHAQ) has decreased MPYS expression in multiple tissues

A–C. Various types of cells from the C57BL/6, HAQ, and MPYS$^{-/-}$ mice were lysed in RIPA buffer and probed for indicated Abs as in Figure 1 (n=3). **D–E.** BMDC or BMDM from the C57BL/6, mHAQ, and MPYS$^{-/-}$ mice were treated with IL-4 (40ng/ml) or IFNγ (40ng/ml) or mock (PBS) overnight. Cells were lysed in RIPA buffer and probed for indicated Abs as in Figure 1 (n=3). **F–G**. BMDC from C57BL/6, MPYS−/− or mHAQ were stimulated with HKSP (10^7c.f.u/ml), LPS (20ng/ml), imiquimod (4ng/ml) or CpG-ODN2395 (8ng/ml) for 5hrs. TNFα **(F)** and IFNβ **(G)** were measured in the cell supernatant (n=3). Graph present means ± SEM from three independent experiments.

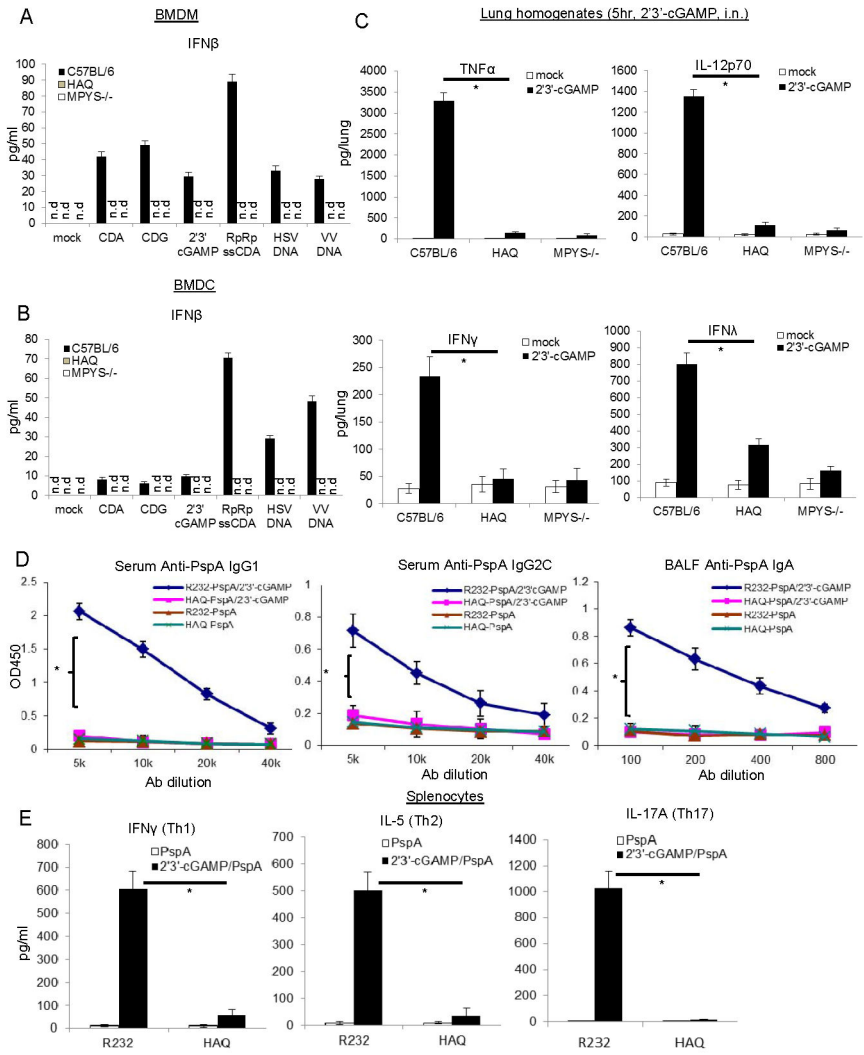

Figure 4. mHAQ mouse does not respond to CDNs in vitro and in vivo

A&B. BMDM or BMDC from C57BL/6, mHAQ and MPYS$^{-/-}$ mice were activated by 10μg/ml of CDA, CDG, 2′3′-cGAMP or 5μg/ml RpRp-ssCDA, HSV-DNA, VV-DNA for 5hrs. Mouse IFNβ was measured in cell supernatant (n=3). **C.** C57BL/6, mHAQ and MPYS$^{-/-}$ mice were treated (*i.n.*) with saline or 2′3′-cGAMP (5μg) for 5hrs. Cytokines were determined in lung homogenates by ELISA (n=3). **D.** WT littermate (R232) and mHAQ mice were immunized (*i.n.*) with PspA (2μg) alone or together with 5μg 2′3′-cGAMP as described in Material and Methods. Anti-PspA IgG1, IgG2C, and IgA were measured by ELISA (n=3). **E.** Splenocytes from PspA or 2′3′-cGAMP + PspA immunized mice were stimulated with 5μg/ml PspA for 4 days in culture. Cytokines were measured in the supernatant by ELISA (n=3). Graph present means ± SEM from three independent experiments. The significance is represented by an asterisk, where $p<0.05$. n.d, not detected.

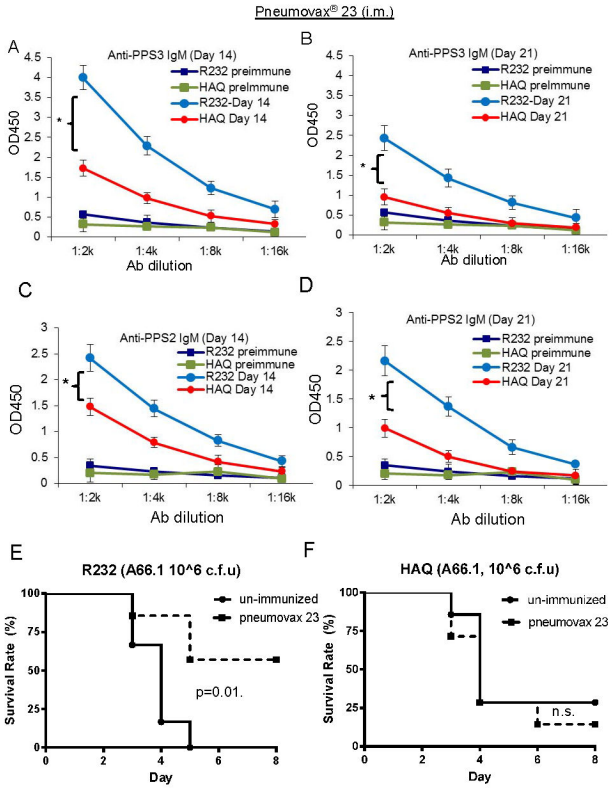

Figure 5. Pneumovax®23 is less effective in mHAQ mice

A–D. WT littermates (R232) and mHAQ mice were immunized (i.m.) with Pneumovax®23 (0.125μg in 50μl saline). Anti-PPS3 (**A–B**) and anti-PPS2 (**C–D**) IgM Ab was determined at day 14 and 21 post immunization as well as pre-immunization (n=3). **E–F**. WT littermates (R232) or mHAQ mice were given (*i.m.*) saline or Pneumovax®23 as in **A–D**. One month post-immunization, mice were infected (*i.n.*) with *S.pneumoniae* (A66.1 strain, ~ 1.0×10^6 c.f.u.). Mice health was monitored for 8 days (n=2). Graph present means ± SEM from three independent experiments. The significance is represented by an asterisk, where $p<0.05$.

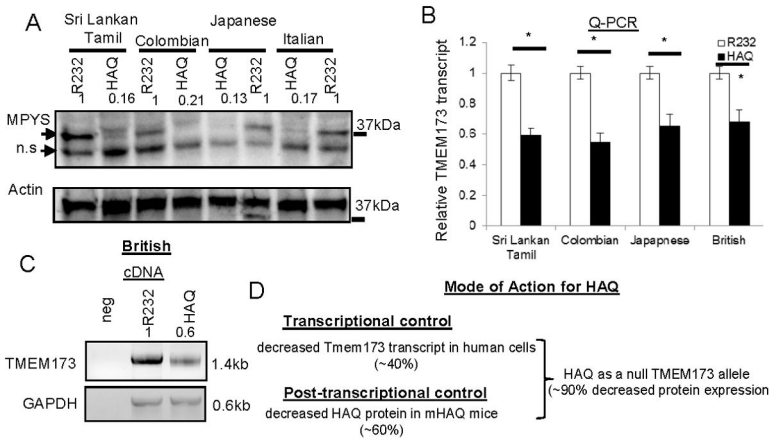

Figure 6. Homozygous *HAQ* human B cells have decreased *TMEM173* transcript

A. MPYS expression was determined by western blot in homozygous *HAQ* or *R232* human B cells from indicated ethnic groups as Figure 1 (n>3). **B**. *TMEM173* mRNA was measured by Q-PCR in the homozygous *HAQ* or *R232* human B cells (n=3). **C**. Full-length human *TMEM173* cDNA was amplified from homozygous *HAQ* and *R232* human B cells as described in Materials and Methods. **D**. Model for the transcriptional and post-transcriptional control of the *HAQ* expression. Graph present means ± SEM from three independent experiments. The significance is represented by an asterisk, where $p<0.05$.

Table 1

Homozygous *HAQ* individuals are common in non-Africans[1].

European		
Genotype	**Carriers**	**Population Frequency**
R232/R232	251	0.499
R232/H232	99	0.1968
HAQ/R232	97	0.1928
HAQ/H232	22	0.0437
H232/H232	14	0.0278
HAQ/HAQ	14	0.0278
AQ/R232	3	0.006
A230/R232	1	0.002
AQ/AQ	1	0.002
AQ/HAQ	1	0.002
Total	**503**	**1**

East Asian		
Genotype	**Carriers**	**Population Frequency**
HAQ/R232	173	0.3433
R232/R232	111	0.2202
HAQ/HAQ	81	0.1607
HAQ/H232	66	0.131
R232/H232	58	0.1151
H232/H232	9	0.0179
AQ/R232	3	0.006
AQ/HAQ	1	0.002
HA/HAQ	1	0.002
AQ/H232	1	0.002
Total	**504**	**1**

African		
Genotype	**Carriers**	**Population Frequency**
R232/R232	255	0.3858
AQ/R232	184	0.2784
R232/H232	88	0.1331
AQ/H232	33	0.0499
AQ/AQ	29	0.0439
Q293/R232	23	0.0348
AQ/Q293	14	0.0212
HAQ/R232	10	0.0152

African		
Genotype	**Carriers**	**Population Frequency**
H232/H232	7	0.0106
Q293/H232	7	0.0106
AQ/HAQ	4	0.0061
HAQ/H232	2	0.003
Q293/Q293	2	0.003
A230-H232/H232	1	0.0015
A230/H232	1	0.0015
AQ/A230	1	0.0015
Total	**661**	**1**

South American		
Genotype	**Carriers**	**Population Frequency**
R232/R232	106	0.3055
HAQ/R232	103	0.2968
R232/H232	56	0.1614
HAQ/HAQ	27	0.0778
HAQ/H232	24	0.0692
H232/H232	11	0.0317
AQ/R232	5	0.0144
HA/HAQ	3	0.0086
AQ/HAQ	3	0.0086
A230/R232	2	0.0058
HAQ/A230	2	0.0058
Q293/R232	2	0.0058
HA/R232	1	0.0029
AQ/H232	1	0.0029
A230/H232	1	0.0029
Total	**347**	**1**

South Asian		
Genotype	**Carriers**	**Population Frequency**
R232/R232	201	0.411
HAQ/R232	159	0.3251
R232/H232	60	0.1227
HAQ/HAQ	33	0.0675
HAQ/H232	28	0.0573
AQ/R232	3	0.0061
H232/H232	3	0.0061
H71/R232	1	0.002
HA/HAQ	1	0.002

South Asian		
Genotype	**Carriers**	**Population Frequency**
Total	**489**	**1**

[1] All *TMEM173* genotypes found in each of the 5 ethnic groups in the 1000Genome Project (Phase III) were summarized. Homozygous *HAQ* individuals are colored in Green. The two other non-functional genotypes: *HAQ/H232* (light yellow) and *H232/H232* (dark yellow) were also colored. Homozygous *HAQ* individuals are absent in African population. Instead, they have the *AQ/AQ* (blue). Notably, heterozygous *HAQ* (*HAQ/R232,* yellow) is the most common *TMEM173* genotype in East Asian population and the second most common genotype in South American and South Asian population.

Table 2

HAQ/HAQ individuals have decreased *TMEM173* transcript in various tissues[1].

R71H			
rs11554776	2.50E-19	−0.7	Artery - Aorta
rs11554776	1.50E-16	−0.44	Artery - Tibial
rs11554776	3.70E-13	−0.45	Cells - Transformed fibroblasts
rs11554776	9.10E-10	−0.33	Thyroid
rs11554776	9.70E-10	−0.44	Lung
rs11554776	1.90E-09	−0.36	Esophagus - Muscularis
rs11554776	1.40E-08	−0.57	Heart - Atrial Appendage
rs11554776	6.50E-08	−0.23	Adipose - Subcutaneous
rs11554776	0.0000012	−0.25	Nerve - Tibial
rs11554776	0.0000047	−0.16	Skin - Sun Exposed (Lower leg)
rs11554776	0.0000092	−0.31	Breast - Mammary Tissue
G230A			
rs78233829	1.50E-22	−0.46	Artery - Tibial
rs78233829	1.10E-17	−0.63	Artery - Aorta
rs78233829	3.70E-15	−0.43	Cells - Transformed fibroblasts
rs78233829	4.50E-11	−0.35	Esophagus - Muscularis
rs78233829	5.60E-11	−0.42	Lung
rs78233829	1.30E-10	−0.31	Thyroid
rs78233829	1.20E-09	−0.23	Adipose - Subcutaneous
rs78233829	6.00E-09	−0.26	Nerve - Tibial
rs78233829	8.60E-09	−0.5	Heart - Atrial Appendage
rs78233829	6.80E-08	−0.17	Skin - Sun Exposed (Lower leg)
rs78233829	0.0000039	−0.28	Pancreas
rs78233829	0.0000054	−0.3	Breast - Mammary Tissue
rs78233829	0.000014	−0.17	Muscle - Skeletal
R293Q			
rs7380824	8.90E-23	−0.45	Artery - Tibial
rs7380824	2.50E-19	−0.66	Artery - Aorta
rs7380824	4.30E-15	−0.42	Cells - Transformed fibroblasts
rs7380824	2.90E-11	−0.35	Esophagus - Muscularis
rs7380824	3.90E-11	−0.42	Lung
rs7380824	1.70E-10	−0.25	Adipose - Subcutaneous
rs7380824	2.80E-10	−0.3	Thyroid
rs7380824	3.50E-09	−0.5	Heart - Atrial Appendage
rs7380824	4.20E-09	−0.26	Nerve - Tibial
rs7380824	6.20E-08	−0.17	Skin - Sun Exposed (Lower leg)

rs7380824	0.0000029	−0.31	Breast - Mammary Tissue
rs7380824	0.000003	−0.29	Pancreas
rs7380824	0.0000062	−0.49	Artery - Coronary
rs7380824	0.0000077	−0.21	Esophagus - Mucosa
rs7380824	0.000009	−0.18	Muscle - Skeletal

[1]Data were compiled from The Genotype-Tissue Expression Database (GTEx).