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The Common R71H-G230A-R293Q Human *TMEM173* Is a Null Allele

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TMEM173 **encodes MPYS/STING and 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. In this study, using data from the 1000 Genomes Project we found that homozygous *HAQ* individuals account for ∼16.1% of East Asians and ∼2.8% of Europeans whereas 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 a decreased *TMEM173* transcript. Consequently, the *HAQ/HAQ* B cells do not respond to CDNs. We subsequently generated an *HAQ* knock-in mouse expressing a 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 dendritic cells, and lung tissue. The mHAQ mouse also does not respond to CDNs in vitro and in vivo. Lastly, Pneumovax 23, with an efficacy that depends on *TMEM173*, is less effective in mHAQ mice than in wild type 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.** ***The Journal of Immunology*****, 2017, 198: 776–787.**

Early detection of invasive pathogens is achieved by germline-encoded innate immune sensors. *TMEM173* encodes an endoplasmic reticulum–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 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 in humans (16, 17). Last, there are ongoing 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 (wild type, 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 nonsynonymous single-nucleotide polymorphisms (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). In this study, we examined the endogenous function of the *HAQ* allele using human cells from homozygous *HAQ* carriers and the knock-in mouse expressing a mouse equivalent of the *HAQ* allele (mHAQ). 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 HAQ-MPYS *knock-in mice*

The linearized targeting vector (Supplemental Fig. 3A), which covers ∼10 kb of the genomic region in *MPYS* locus on mouse chromosome 18, was transfected into JM8A3.N1 embryonic stem cells originated from the C57BL/6 strain, followed by the selection for neomycin-positive and diphtheria toxin–negative clones. Targeted clones were screened by PCR. Positive embryonic stem 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 (Supplemental Fig. 3B). The heterozygous mice were bred to Actin-flpase mice [The Jackson Laboratory, B6.Cg-Tg(ACTFLPe)9205Dym/J] (Supplemental Fig. 3A) 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.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMDC, BM-derived dendritic cell; BMDM, BM-derived macrophage; CDA, cyclic di-AMP; CDG, cyclic di-GMP; CDN, cyclic dinucleotide; 2′3′-cGAMP, 2′5-3′5′-cyclic GMP-AMP; GTEx, Genotype-Tissue Expression; *HAQ*, R71H-G230A-R293Q; HKSP, heat-killed *Streptococcus pneumoniae*; i.n., intranasally; mHAQ, a knock-in mouse expressing the mouse equivalent of the human HAQ; PPS2, pneumococcal polysaccharide type 2; PPS3, pneumococcal polysaccharide type 3; PspA, pneumococcal surface protein A; Q-PCR, quantitative PCR; RIPA, radioimmunoprecipitation assay; SNP, single-nucleotide polymorphism; WT, wild type.

Mice

For all experiments, 6- to 12-week old mice, both males and females, were used. MPYS$^{-/-}$ mice (Tmem173$^{<tm1Camb>}$) have been 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 following 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, National Institute of Allergy and Infectious Diseases, National Institutes of Health: *Streptococcus pneumoniae* 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 the 1000 Genomes 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, 2 mM L-glutamine, 37˚C under 5% CO_2. Information related to *TMEM173* gene expression was obtained from the Genotype-Tissue Expression (GTEx) project (http://www.gtexportal.org/home/).

Human B cell activation by CDNs

For CDA (cat#vac-cda; Invivogen), CDG (cat#vac-cdg; Invivogen), and 2′3′-cGAMP (cat#vac-cga23; Invivogen) activation, human B cells were harvested and suspended (5×10^6 cells/ml) in transporter buffer (26) (110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, 20 mM HEPE pH 7.3 and protease-inhibitor mixture (cat# B14011; BioTool)) with 10 μg/ml digitonin (cat# 300410; Calbiochem) in the presence or absence of CDNs (10 μg/ml). Cells were cultured at 37˚C for 10 min in 24-well plates. 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 (10 μg/ml) for 5 h. Human IFN-β was measured in cell supernatant by ELISA (cat#41415; PBL Bioscience).

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

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

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

Quantitative PCR to determine TMEM173 *mRNA in human B cells*

Human B cells (1.2×10^6) were harvested and lysed in 350 μl of RLT sample buffer with 40 μM DTT. Total RNA was extracted using the RNeasy Plus Mini kit (cat#74134; Qiagen) and reverse-transcribed using the high capacity reverse transcription kit (Applied Biosystems). Quantitative PCR (Q-PCR) was carried out on a StepOnePlus 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* (Assay ID: qHsaCID0010565, cat# 10025636; BioRad). 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 (cat#74134; Qiagen). Total cDNA was made using the Superscript IV First-Strand Synthesis System (#18091050; Invitrogen). Full-length human *TMEM173* gene (1379 bp) was amplified with following primers: *TMEM173-For*: 5′-TTGGCTGAGTGTGTGGAGTC-3′; *TMEM173-Rev*: 5′-CAGTCCAGAGGCTTGGAGAC-3′. Human *GAPDH* primers (# RDP39; R&D Systems) were used to amplify the *GAPDH* cDNA as a control.

Bone marrow–derived macrophage and bone marrow–derived dendritic cell activation

Bone marrow (BM) cells were cultured in RPMI 1640 (cat#11965; Invitrogen) with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 1% nonessential amino acids, 50 μM 2-ME, 1% Pen/Strep, 20 ng/ml GM-CSF (cat# RP0407M; Kingfisher) or 20 ng/ml M-CSF (cat# RP0462M; Kingfisher). 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 10 μg/ml CDA, CDG, 2′3′-cGAMP or 5 μg/ml Rp-Rp-ssCDA in culture directly. Mouse IFN-β was measured in culture supernatant after 5 h by ELISA (cat#42410; PBL Bioscience). Separately, BM-derived macrophages (BMDM) and BM-derived dendritic cells (BMDC) were activated with 5 μg/ml HSV DNA (cat# tlrl-hsv60n; Invivogen) and Vaccinia virus DNA (cat# tlrl-vav70n; Invivogen) transfected with lipofectamine 2000 (27) and mouse IFN-β was measured in culture supernatant after 5 h by ELISA. Alternatively, BMDC were activated with heat-killed *S. pneumoniae* (HKSP) (10^8 CFU/ml) (cat# tlrl-hksp; Invivogen), LPS from *Salmonella* (25 ng/ml) (cat# L7261; Sigma), Imiquimod (4 ng/ml) (cat# tlrl-imqs; Invivogen), or CpG-ODN2395 (8 ng/ml) (cat# tlrl-2395; Invivogen). Mouse TNF-α and IFN-β were measured in culture supernatant after 5 h by ELISA.

In vivo CDN activation

Mice were intranasally (i.n.) administered 5 μg 2′3′-cGAMP (cat#vac-cga23; Invivogen), then sacrificed after 5 h by CO_2 asphyxiation (11). Lungs were perfused with cold PBS. The harvested lungs were washed once with PBS, then stored in 0.7 ml tissue protein extraction reagent (cat#78510; Thermo Scientific) containing protease inhibitors (cat#11836153001; Roche) at −80˚C. Later, the lung was thawed on ice and homogenized with Minilys (Precellys, 5000 RPM for 30 s) using a Precellys lysing kit (cat# KT03961; Precellys). Lung homogenates were transferred to a 1.5 ml tube and spun at 14,000 g for 30 min 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 (four per group) were vaccinated i.n. with 5 μg 2′3′-cGAMP adjuvanted PspA (2 μg; BEI Resources) or PspA alone (11). Mice were immunized twice at a 14 d interval. For intranasal vaccination, animals were anesthetized using isoflurane in an E-Z Anesthesia system (Euthanex, Palmer, PA). PspA, with or without 2′3-cGAMP, was administered in 20 μl saline. Sera, bronchoalveolar lavage fluid, and nasal washes were collected 14 d after the last immunization. The PspA-specific Abs were determined by ELISA. Secondary Abs used were anti-mouse IgG1-HRP (cat#1070-05; Southern Biotech), anti-mouse IgG2C-HRP (cat#1079-05; Southern Biotech), and anti-mouse IgA-HRP (cat#1040-05; Southern Biotech). To determine the Ag-specific Th response, splenocytes from PspA or 2′3-cGAMP + PspA immunized mice were stimulated with 5 μg/ml PspA for 4 d in culture. Th1, Th2, and Th17 cytokines were measured in the supernatant by ELISA.

Pneumovax 23 immunization

A group of mice (4 mice per group for the Ab experiment and 10 mice per group for the survival experiment) was i.m. administered with 0.125 μg of Pneumovax 23 (cat#7002681601; Merck) in 50 μl Ultrapure PBS (cat#K812; Amresco) or PBS alone. Blood was collected before and after immunization at the indicated time. Anti-pneumococcal polysaccharide type 2 (PPS2) and pneumococcal polysaccharide type 3 (PPS3) IgM were determined by ELISA. The following reagents were used: PPS3 (ATCC 31-X, ID # 61810463), PPS2 (ATCC 500-X, ID# 63406999), 1× ELISA assay diluent (REF # 00-4202-43; eBioscience), and goat anti-Mouse IgM HRP (cat # 1020-05; SouthernBiotech). One month after immunization, mice were challenged (i.n) with *S. pneumoniae* (A66.1 strain, serotype 3, ∼10^6 CFU in 50 μl PBS). Animal health was monitored for 8 d.

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 three 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) 5′-GGAGTGACACACGTTGG-3′, R-primer (Rs11554776 A) 5′-GCCTAGCTGAGGAGCTG-3′, probe (rs11554776 C): 5′-CTGGAGTGGA-3′-XI-5′-TGTGGCGCAG-3′-PH. Primer and probes for the *TMEM173* SNP R293Q were as follows: F-primer (rs7380824 F): 5′-ACCCTGGTAGG-CAATGA-3′, R-primer (rs7380824 R): 5′-GCTTAGTCTGGTCTTCCTCTTAC-3′, sensor probe (rs7380824 C): 5′-CCTCAAGTGTCCGGCAGAAGAGTT-3′-FL, anchor probe (Anc rs7380824): 640-5′-GGCCTGCTCAAGCCTATCCTCC-CGG-3′-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). 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) and reverse-transcribed using the high capacity reverse transcription kit (Applied Biosystems). Q-PCR was carried out on an ABI 7300 instrument (Applied Biosystems) using the following primers and probes: ifnb: F-primer: 5′-CCAACAAGTGTCTCCTCCAAATT-3′, R-primer: 5′-GTAGGAATCCAAGCAAGTGTAGCT-3′, probe: FAM-5′-TGTTGTGC-TTCTCCACTACAGCTCTTTCCA-3′-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 mean ± SEM. Statistical significance was evaluated using Prism 5.0 software to perform a Student *t* test (unpaired, two-tailed) for comparison of mean values.

Results

Homozygous HAQ *individuals are common among non-Africans*

We previously estimated that ∼3% of Americans are *HAQ/HAQ* (23). To expand this to other ethnic groups, we extracted *TMEM173* genotype data from the 1000 Genomes Project (phase III). Among

Table I. Homozygous *HAQ* individuals are common among non-Africans[a]

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
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
Total	**489**	**1**

[a]All *TMEM173* genotypes found in each of the five ethnic groups in the 1000 Genomes Project (phase III) were summarized. Homozygous *HAQ* individuals are colored in bright green. The two other nonfunctional genotypes, *HAQ/H232* (light green) and *H232/H232* (orange), were also colored. Homozygous *HAQ* individuals are absent in the African population. Instead, they have the *AQ/AQ* (blue). Notably, heterozygous *HAQ* (*HAQ/R232*, bright yellow) is the most common *TMEM173* genotype in the East Asian population and the second most common genotype in South American and South Asian population.

the five ethnic groups defined in the 1000 Genomes Project, we found that *HAQ/HAQ* is most common in East Asians (~16.07%), followed by South Americans (~7.78%), South Asians (~6.75%), and Europeans (~2.78%) (Table I). Surprisingly, no homozygous *HAQ* individual was found among Africans (Table I). Instead, ~4.39% of Africans are *AQ/AQ* (G230A-R293Q), which is not found in non-Africans (Table I). We concluded that the human *TMEM173* gene has not only great heterogeneity but also shows significant population stratification.

Homozygous HAQ *B cells have very low MPYS protein expression compared with* R232 *B cells*

To study the function of *HAQ*, we obtained EBV-transformed human B cells from homozygous *HAQ* individuals identified in

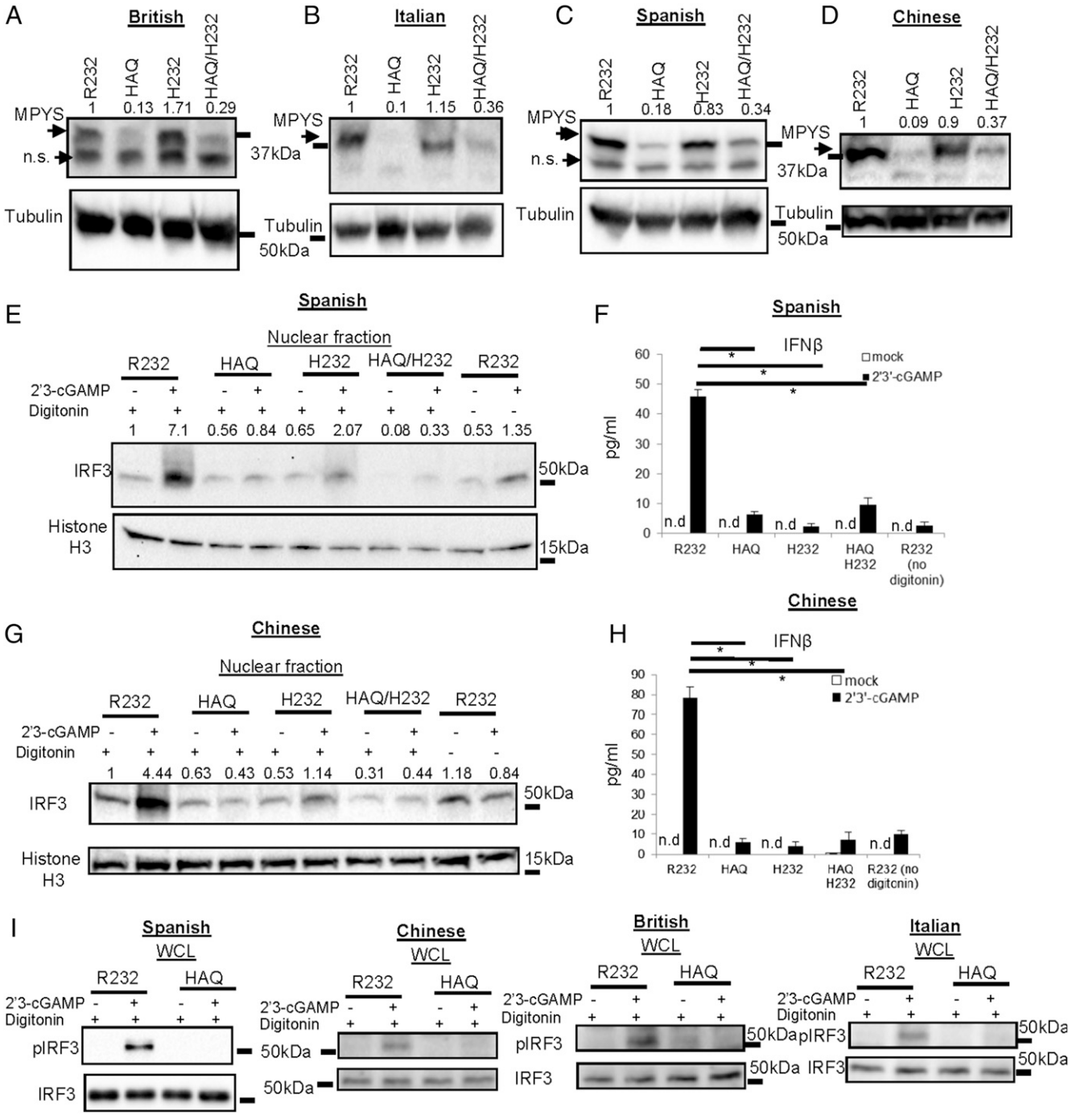

FIGURE 1. Homozygous *HAQ* human B cells have very low MPYS protein expression compared with the *R232* B cells and do not respond to natural CDNs. (**A**–**D**) *R232/R232*, *HAQ/HAQ*, *H232/H232*, and *HAQ/H232* human B cells from indicated ethnic groups were lysed in RIPA buffer and probed for MPYS expression using the rabbit anti-mouse MPYS Ab ($n > 3$). (**E** and **G**) *R232/R232*, *HAQ/HAQ*, *H232/H232*, and *HAQ/H232* human B cells from indicated ethnic groups were activated with 2′3′-cGAMP (10 μg/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** and **H**) Human IFN-β was measured in cell supernatant from (E and G) by ELISA ($n = 3$). (**I**) *R232/R232*, *HAQ/HAQ* human B cells from indicated ethnic groups were activated with 2′3′-cGAMP (10 μg/ml) for 5 h as in (E and 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 ($n = 2$). Graphs present means ± SEM from three independent experiments. The significance is represented by $*p < 0.05$. n.s, nonspecific. n.d, not detected.

the 1000 Genomes Project. These cells are distributed by National Human Genome Research Institute Repository at the Coriell Institute. They express B cell surface markers IgM and HLA-DR (Supplemental Fig. 1A). Notably, these cells also express B cell activation markers CD80, CD86, and CD69 (Supplemental Fig. 1A). The expression level of these markers is similar between *R232* (WT) and HAQ B cells (Supplemental Fig. 1A).

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 (Fig. 1A–D). No MPYS protein was detected in the cell debris (Supplemental Fig. 1B) 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 in 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*

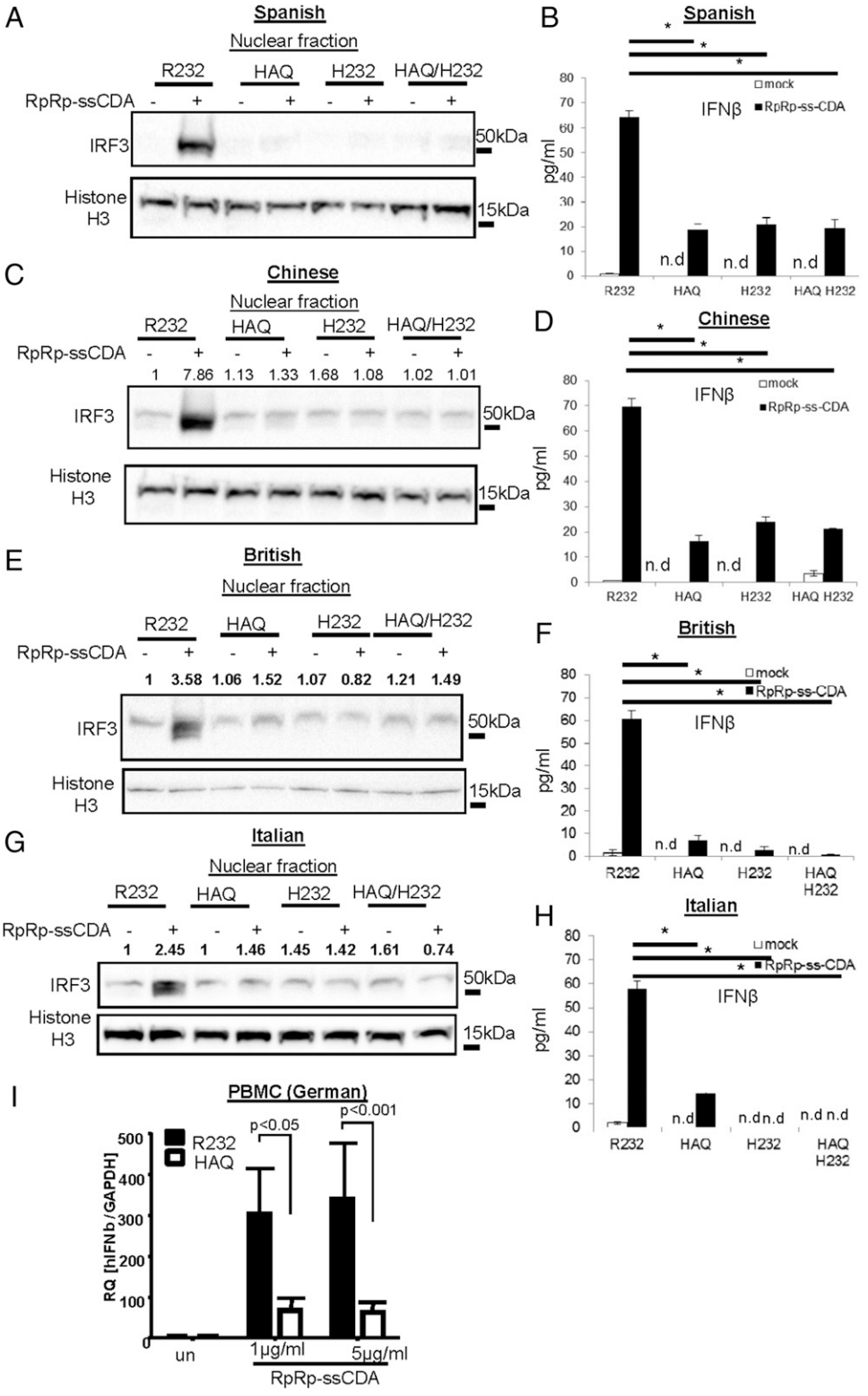

FIGURE 2. Homozygous *HAQ* human B cells are defective in response to synthetic CDN RpRp-ssCDA. (**A**, **C**, **E**, and **G**) *R232/R232*, *HAQ/HAQ*, *H232/H232,* and *HAQ/H232* human B cells from indicated ethnic groups were activated with RpRp-ssCDA (5 μg/ml) for 5 h in culture. Nuclear fractions were isolated, run on a SDS-PAGE gel and probed with the indicated Abs ($n = 3$). (**B**, **D**, **F**, and **H**) Human IFN-β was measured in cell supernatant from (A, C, E, and G) by ELISA ($n = 3$). (**I**) PBMCs from three homozygous *HAQ* and *R232* Germans were stimulated with RpRp-ssCDA. Relative expression of *ifnb* was determined by Q-PCR ($n = 3$). Graphs present means ± SEM from three independent experiments. The significance is represented by $*p < 0.05$. n.d, not detected.

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 (Supplemental Fig. 1C), which indicated that our anti-MPYS Ab recognizes the HAQ of MPYS is as good as the R232 of MPYS. We thus concluded that the low MPYS staining in the *HAQ* B cells (Fig. 1A−D) 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 (Supplemental Fig. 1C), 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 low MPYS expression.

Directly adding CDN to the human B cell cultures did not activate these cells (Fig. 1E, 1G, Supplemental Fig. 2A, 2C, 2E, 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 IFN-β production. In Spanish and Chinese samples, 2′3′-cGAMP activates IRF3 translocation and IFN-β production in *R232/R232* individuals but not the *HAQ/HAQ* B cells (Fig. 1E–H). Furthermore, 2′3′-cGAMP did not induce IRF3 phosphorylation in the *HAQ/HAQ* cells from the Chinese, Spanish, British, or Italian samples (Fig. 1I). Similar observations were made in *HAQ/HAQ* samples in response to CDA and CDG (Supplemental Fig. 2A–F).

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

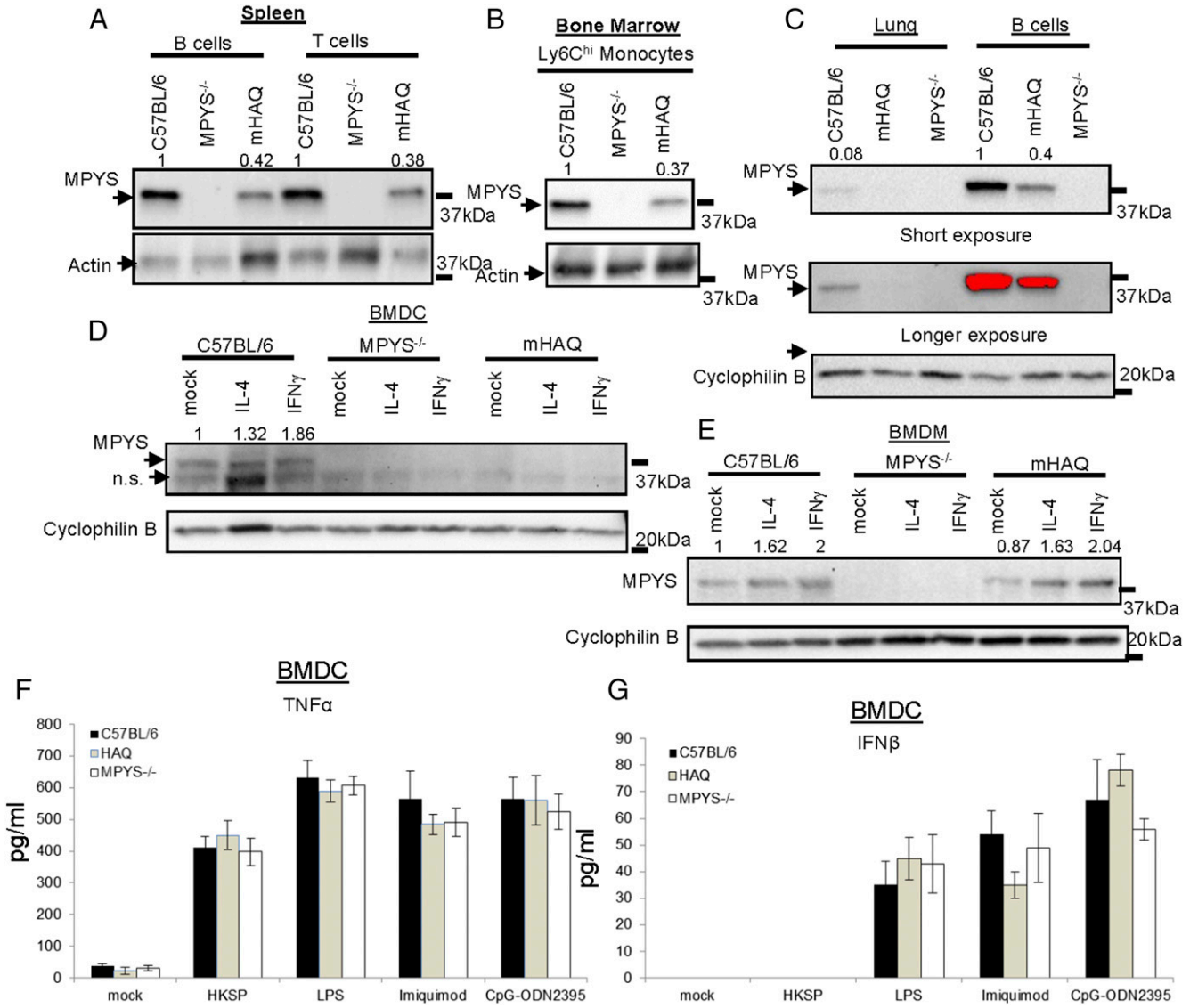

FIGURE 3. The mHAQ mouse has a decreased MPYS expression in multiple tissues. (**A**–**C**) Various types of cells from the C57BL/6, mHAQ, and MPYS$^{-/-}$ mice were lysed in RIPA buffer and probed for indicated Abs as in Fig. 1 (n = 3). (**D** and **E**) BMDC or BMDM from the C57BL/6, mHAQ, and MPYS$^{-/-}$ mice were treated with IL-4 (40 ng/ml) or IFN-γ (40 ng/ml) or mock (PBS) overnight. Cells were lysed in RIPA buffer and probed for indicated Abs as in Fig. 1 (n = 3). (**F** and **G**). BMDC from C57BL/6, MPYS$^{-/-}$ or mHAQ were stimulated with HKSP (10^7 CFU/ml), LPS (20 ng/ml), imiquimod (4 ng/ml) or CpG-ODN2395 (8 ng/ml) for 5 h. TNF-α (F) and IFN-β (G) were measured in the cell supernatant (n = 3). Graphs present means ± SEM from three independent experiments.

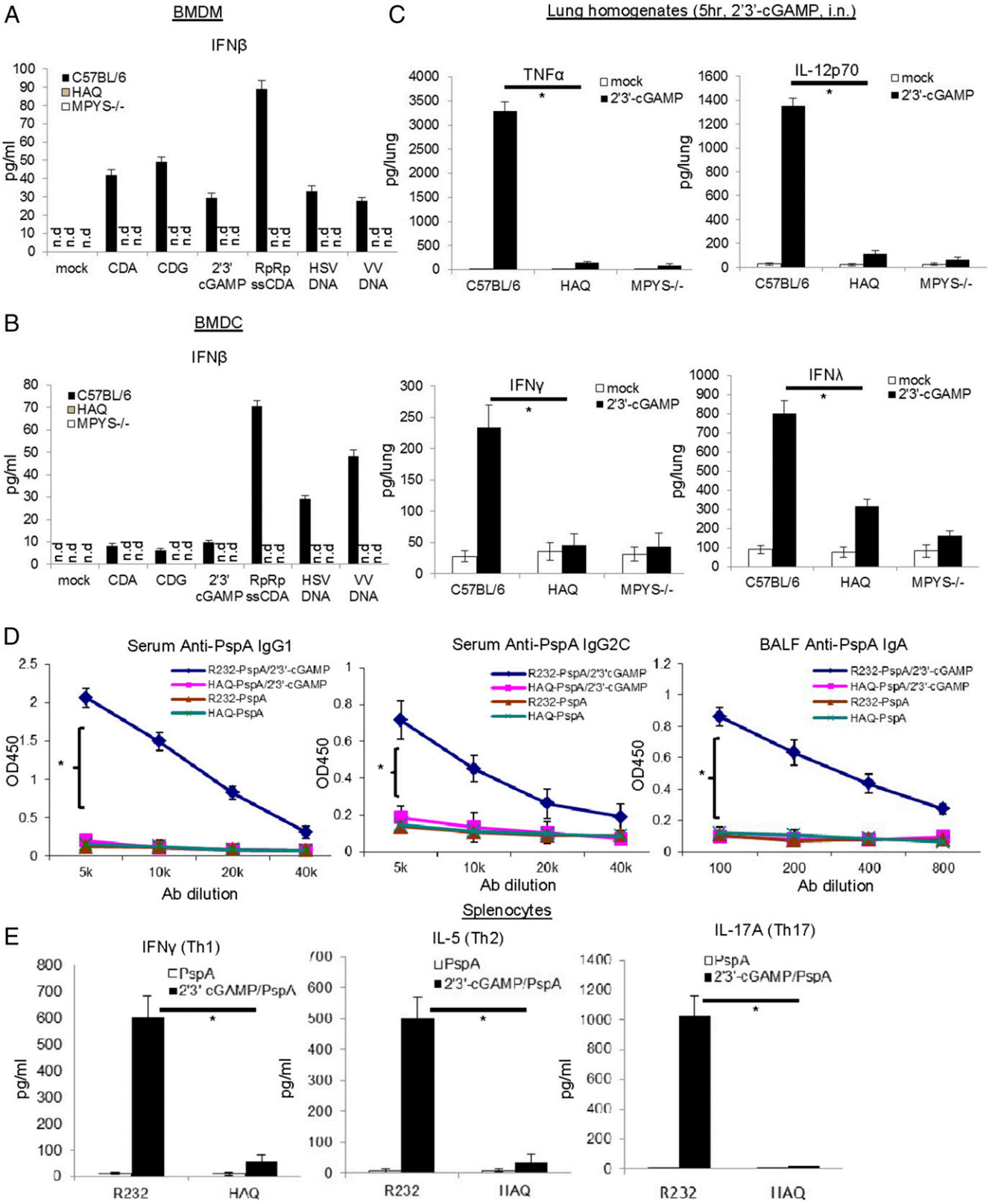

FIGURE 4. mHAQ mouse does not respond to CDNs in vitro and in vivo. (**A** and **B**) BMDM or BMDC from C57BL/6, mHAQ, and MPYS$^{-/-}$ mice were activated by 10 μg/ml of CDA, CDG, 2′3′-cGAMP, or 5 μg/ml RpRp-ssCDA, HSV-DNA, VV-DNA for 5 h. Mouse IFN-β was measured in cell supernatant (n = 3). (**C**) C57BL/6, mHAQ, and MPYS$^{-/-}$ mice were treated (i.n.) with saline or 2′3′-cGAMP (5 μg) for 5 h. Cytokines were determined in lung homogenates by ELISA (n = 3). (**D**) WT littermate (R232) and mHAQ mice were immunized (i.n.) with PspA (2 μg) alone or together with 5 μg 2′3′-cGAMP as described in *Materials 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 5 μg/ml PspA for 4 d in culture. Cytokines were measured in the supernatant by ELISA (n = 3). Graphs present means ± SEM from three independent experiments. The significance is represented by $*p < 0.05$. n.d, not detected.

RelB (Supplemental Fig. 1D). CDN activation, which increases nuclear IRF3, did not further increase nuclear RelA or RelB (Supplemental Fig. 1D). This is consistent with our observation that these B cells have an activated phenotype (Supplemental Fig. 1A).

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

Recently, a synthetic CDN, RpRp-ssCDA, was shown 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 a medium without the need of permeabilization (Fig. 2A, 2C, 2E, 2G). Second, *HAQ/HAQ* cells from British, Italian, Spanish, and Chinese individuals are all defective in IRF3 activation and IFN-β production in response to RpRp-ssCDA (Fig. 2A–H). Lastly, PBMC from three German *HAQ/HAQ* individuals also had a defective IFN-β response to RpRp-ssCDA compared with the *R232/R232* individuals (Fig. 2I). RpRp-ssCDA can induce Type I IFN production in the Goldenticket mouse, which lacks detectable MPYS/STING protein (18); this may explain the residual IFN-β 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* HAQ/H232 *human B cells are defective in response to natural and synthetic CDNs*

The H232 of MPYS has a low binding affinity for CDNs (6). We found that MPYS expression in *H232/H232* human B cells is similar to *R232/R232* whereas the *HAQ/H232* B cells have decreased MPYS expression likely due to the presence of the *HAQ* allele (Fig. 1A–D). 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 IFN-β production in response to 2′3′-cGAMP, CDA, CDG or RpRp-ssCDA (Fig. 1E–H, Fig. 2, Supplemental Fig. 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.

Establishing 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 mHAQ mouse. This knock-in mouse contains a mouse

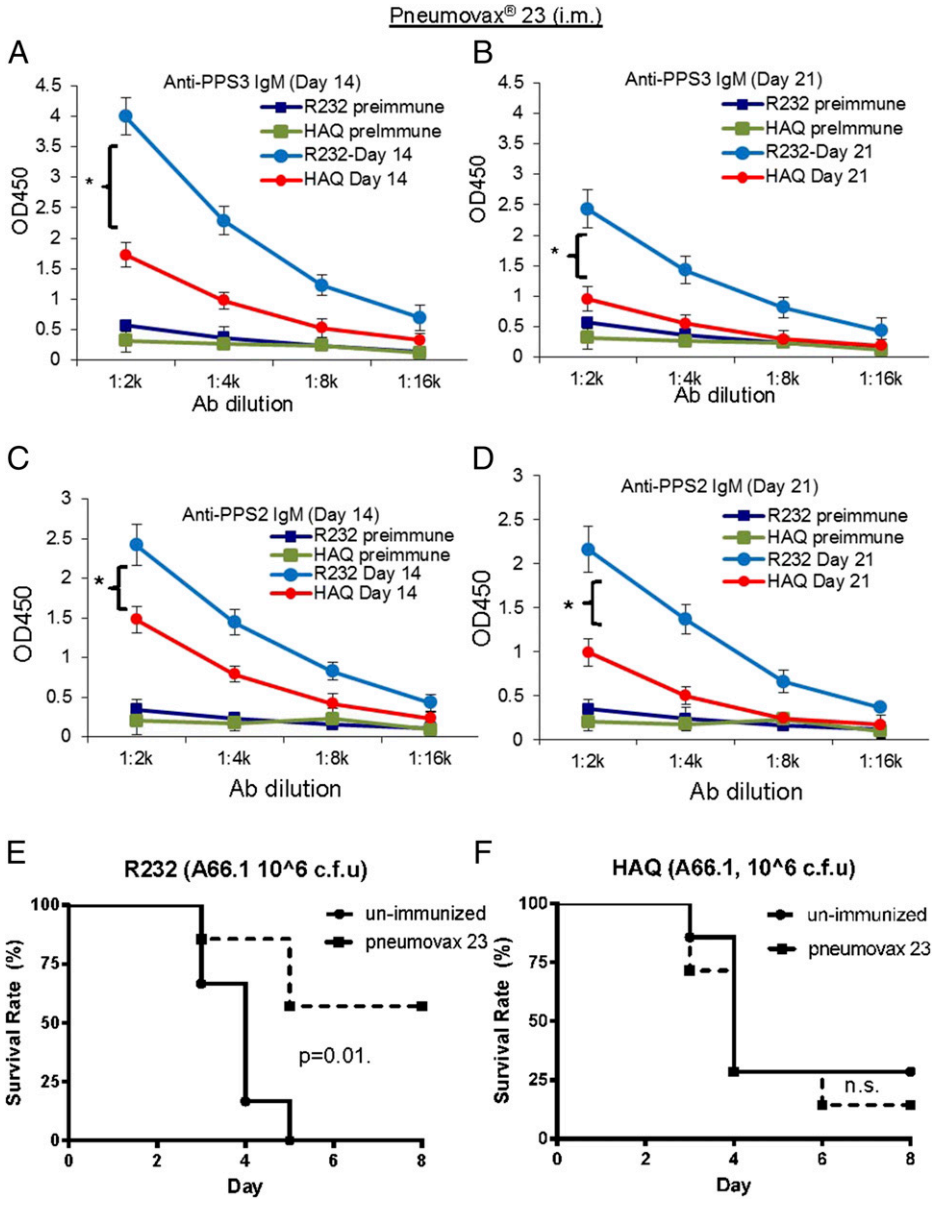

FIGURE 5. Pneumovax 23 is less effective in mHAQ mice. (**A**–**D**). WT littermates (R232) and mHAQ mice were immunized (i.m.) with Pneumovax 23 (0.125 μg in 50 μl saline). Anti-PPS3 (A and B) and anti-PPS2 (C and D) IgM Ab was determined at day 14 and 21 post-immunization as well as preimmunization (n = 3). (**E** and **F**) WT littermates (R232) or mHAQ mice were given (i.m.) saline or Pneumovax 23 as in (A–D). One month postimmunization, mice were infected (i.n.) with *S. pneumoniae* (A66.1 strain, ~1.0 × 10^6 CFU). Mice health was monitored for 8 d (n = 2). Graphs present means ± SEM from three independent experiments. The significance is represented by $*p < 0.05$.

equivalent of the *HAQ* mutations: C71H, I229A, and R292Q (Supplemental Fig. 3A). The presence of these three mutations was confirmed by sequencing (Supplemental Fig. 3B). Similar to the human *HAQ* B cell, we found that MPYS expression is also decreased in mHAQ spleen B cells (Fig. 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 (Fig. 3A) and mHAQ BM Ly6C^{hi} monocytes (Fig. 3B). The defect is more pronounced in the mHAQ lung (Fig. 3C) and mHAQ BMDC (Fig. 3D) where MPYS expression is not detectable. Notably, in naive BMDM, IFN-γ differentiated M1 macrophage or IL-4 differentiated M2 macrophage, MPYS expression is similar for WT and mHAQ mice (Fig. 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 nonresponsive to CDNs (Fig. 1, Supplemental Fig. S2). We next examined CDNs response in BMDC and BMDM from the mHAQ mouse. As expected, both mHAQ BMDM and BMDC did not produce IFN-β in response to CDA, CDG, 2′3′-cGAMP, or RpRp-ssCDA (Fig. 4A, 4B). The mHAQ BMDM and BMDC also did not make IFN-β in response to transfected HSV DNA or Vaccinia virus DNA (Fig. 3A, 3B). As a control, BMDC from mHAQ mice had similar TNF-α (Fig. 3F) and IFN-β (Fig. 3G) production as the WT mice when stimulated with TLR2 ligand 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 production in the lung, which 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 TNF-α, IL-12p70, IFN-γ, or IFN-λ in the mHAQ mouse (Fig. 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 (Fig. 4D, 4E). We concluded that the mHAQ mouse does not respond to CDN in vivo and in vitro.

Pneumovax 23 is less effective in mHAQ mice than in WT mice

The CDNs-MPYS/STING activation in B cells is required for polysaccharide-based vaccine activity such as Pneumovax 23 (33). Because mHAQ mice do not have a functional CDNs-MPYS pathway (Fig. 4), we hypothesized that Pneumovax 23 would not be effective in the mHAQ mouse. Indeed, upon i.m. Pneumovax 23 immunization, the mHAQ mice have lower anti-PPS3 IgM (Fig. 5A, 5B) and anti-PPS2 IgM (Fig. 5C, 5D) production than the WT mice at days 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 *Streptococcus pneumoniae*. Consistent with the Ab results, Pneumovax 23 protected WT mice from the A66.1 *S. pneumococcus* infection (Fig. 5E) but not the mHAQ mouse (Fig. 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 individuals, which have low MPYS expression (Fig. 6A), all have ∼40% lower *TMEM173* mRNA than their *R232/R232* ethnic controls (Fig. 6B). Semiquantitative PCR also show that the full-length human *TMEM173* transcript (∼1.4 kb) is decreased in homozygous *HAQ* B cells compared with their *R232* counterparts (Fig. 6C). We concluded that human *HAQ* B cells 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 GTEx database, which compiles data on human gene expression related to genetic

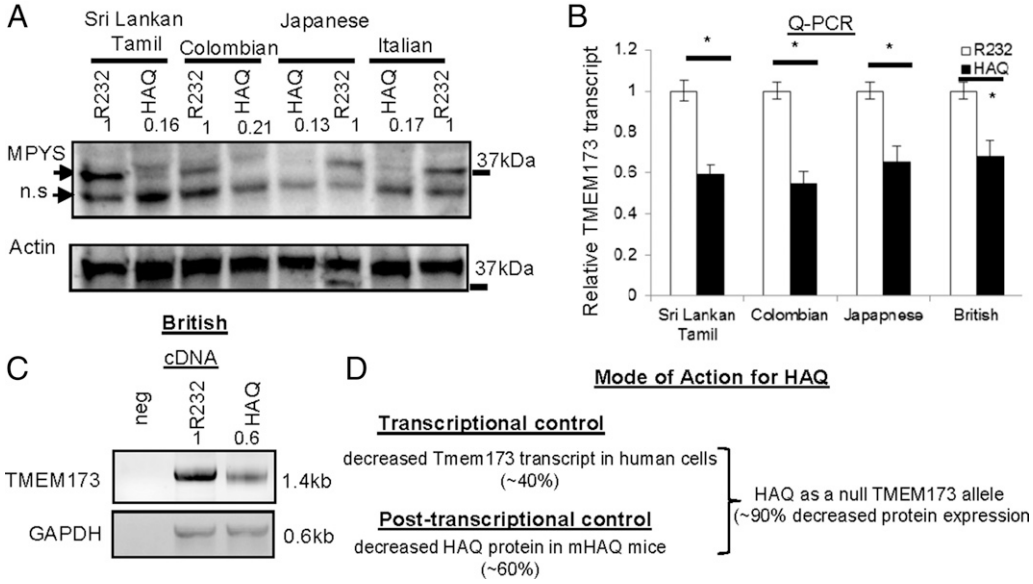

FIGURE 6. Homozygous *HAQ* human B cells have decreased *TMEM173* transcript. (**A**) MPYS expression was determined by western blot in homozygous *HAQ* or *R232* human B cells from indicated ethnic groups as Fig. 1 ($n > 3$). (**B**) *TMEM173* mRNA was measured by Q-PCR in the homozygous *HAQ* or *R232* 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 posttranscriptional control of the *HAQ* expression. Graphs present means ± SEM from three independent experiments. The significance is represented by $*p < 0.05$.

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 II). Furthermore, this decreased *TMEM173* transcript in *HAQ* individuals can be found in non–B cell dominant tissues such as in the artery, fibroblasts, lung, thyroid and esophagus (Table II). 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 IFN-β production when transiently overexpressed in the 293T cells, a hallmark function of MPYS/STING (23). In 2013, Diner et al. (5) found that the THP-1 cell, a human monocytic cell line originated from a Japanese individual (34), has the *HAQ* of *TMEM173*. However, it is not clear if the THP-1 cells are homozygous or heterozygous for *HAQ*. Also in 2013, Yi et al. (24) found that 293T cells stably expressing the *HAQ* can respond to CDN, albeit weaker than the *R232* of *TMEM173*. Using 293T cell stable transfectants to study *HAQ* function, nevertheless, could be misleading. This is because: 1) the level of MPYS is 50-fold higher in the 293T cell transfectants than the endogenous MPYS (Supplemental Fig. 1C); and 2) human *HAQ* cells have a decreased *TMEM173* transcript (Fig. 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, which showed that homozygous *HAQ* B cells have very low MPYS expression compared with the *R232* B cells and do not respond to CDN in vitro. Furthermore, PBMCs from three homozygous *HAQ* Germans have a decreased CDN response compared with 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 (Figs. 1, 2). The underlying molecular mechanisms are likely different. Unlike the *HAQ/HAQ*, the *H232/H232* B cells have similar MPYS expression as the *R232/R232* B cells. Previous studies found that the H232 of MPYS does not bind CDN (K_d ~5.3 μM) as well as the R232 of MPYS (K_d ~0.11 μM) (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

Table II. *HAQ/HAQ* individuals have decreased *TMEM173* transcript in various tissues[a]

SNP ID	*p* Value	Effect Size	Tissue
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

[a]Data were compiled from GTEx.

H232 human B cells (Fig. 1). The *HAQ/H232* B cells have the *HAQ* allele, which contributes to its low MPYS expression (Fig. 1), and the nonfunctional *H232* allele. Together, they lead to the unresponsiveness to CDN in the *HAQ/H232* B cells.

It is worth noting that the *HAQ/HAQ*, *HAQ/H232,* and *H232/H232* genotypes are made up of ∼10% Europeans and ∼31% East Asians (Table I). This is significant because there is tremendous interest in developing 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 have showed that the licensed pneumococcal vaccine Pneumovax 23 is less effective in the mHAQ mouse than in WT mice (Fig. 5). Fu et al. (18) did show that the synthetic CDN RpRp-ssCDA activates PBMCs similarly in *HAQ/HAQ* and *R232/R232* donors. However, the PBMC was from a single *HAQ/HAQ* donor (18). In this study, we used three homozygous German *HAQ* individuals and found they were defective in response to RpRp-ssCDA (Fig. 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 them 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.

Finally, 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 (Fig. 6D). The MPYS protein level is down ∼60% in the mHAQ knock-in mouse. Previously, an I199N change in the mouse *TMEM173* gene led to a complete loss of 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 a STAT-1 dependent mechanism (37). Nevertheless, treating homozygous human *HAQ* B cells with IFN-γ 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 posttranscriptional 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, is not protected by Pneumovax 23. Future studies are needed to determine the impact and mechanisms by which *HAQ*, as a loss-of-function common *TMEM173* allele, influences human diseases, and medicines. Our m*HAQ* knock-in mouse will be especially valuable in this endeavor.

Disclosures

The authors have no financial conflicts of interest.

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Corrections

Patel, S., S. M. Blaauboer, H. R. Tucker, S. Mansouri, J. S. Ruiz-Moreno, L. Hamann, R. R. Schumann, B. Opitz, and L. Jin. 2017. The common R71H-G230A-R293Q human *TMEM173* is a null allele. *J. Immunol*. 198: 776–787.

The amino acid labels in the originally published Supplemental Fig. 3 indicated their locations in the *HAQ* knock-in mouse of an equivalent human *HAQ*. To avoid confusion, we have changed these labels from A230 (human STING) to A229 (mouse STING), and from Q293 (human STING) to Q292 (mouse STING), and this replacement figure has been published online. We have also provided new sequencing results for the A229 and Q292 changes in the mHAQ mouse. The current online supplemental figure therefore differs from what was originally published online.

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Supplementary Data

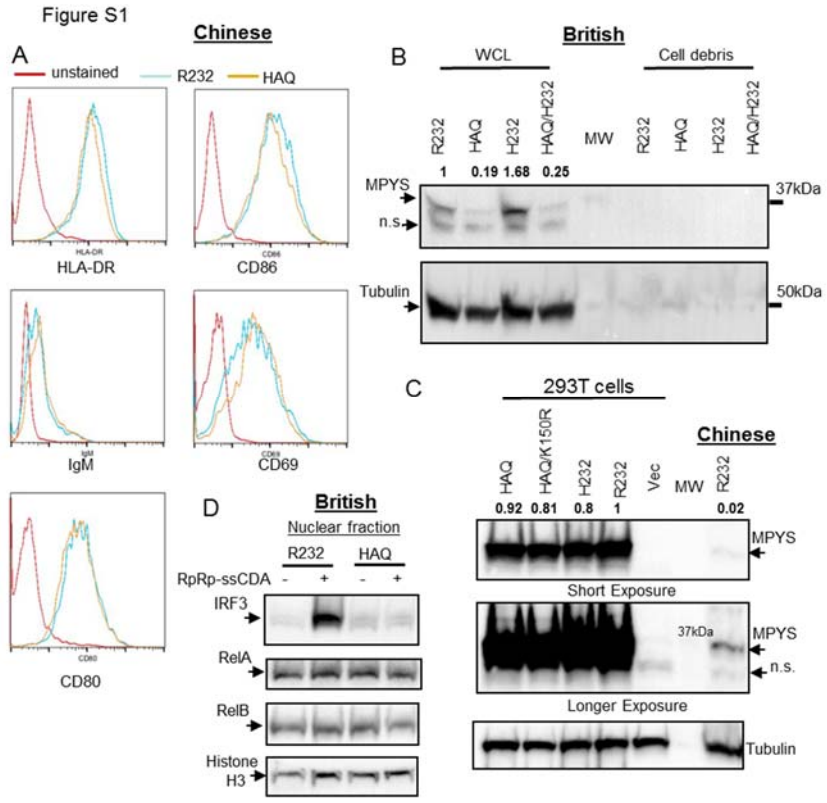

Figure S1. EBV-transformed human B cells express MPYS. A. *R232/R232* and *HAQ/HAQ* human B cells (Chinese) were stained with indicated Abs. Live cells were analyzed by flow cytometry (n = 2). **B**. *R232/R232, HAQ/HAQ, H232/H23, HAQ/H232* human B cells samples (British) were lysed in RIPA buffer. WCL and cell debris were separated by centrifuge (14,000g for 10mins), run on a SDS-PAGE gel, and probed for indicated proteins (n=2). **C.** Parent 293T cells, 293T cells stably transfected with indicated human *TMEM173* variants and *R232/R232* human B cells (Chinese) were lysed in RIPA buffer. WCL was run and probed for indicated proteins (n=3). **D**. *R232/R232, HAQ/HAQ* human B cells (British) were activated with RpRp-ssCDA (5μg/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). n.s. nonspecific. M.W. molecular weight marker.

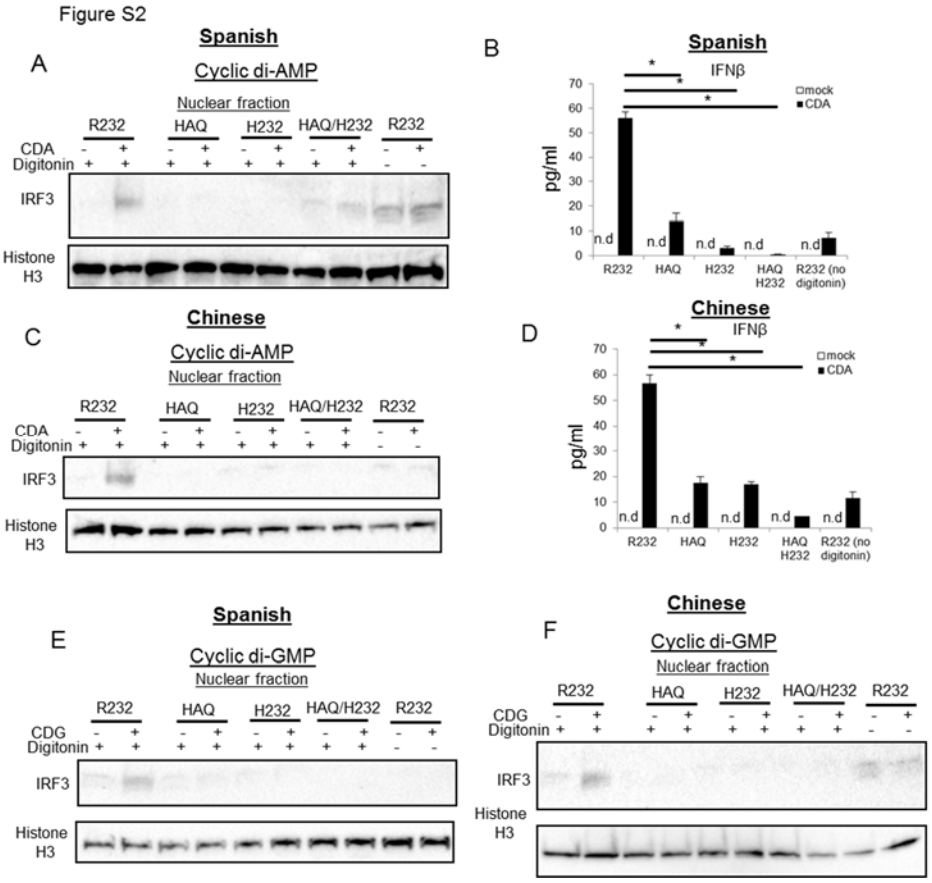

Figure S2. Homozygous *HAQ* B cells do not respond to CDNs. A&C. *R232/R232, HAQ/HAQ, H232/H232 and HAQ/H232* human B cells from indicated ethnic groups were activated with CDA (10 μg/ml) for 5 h as described in Materials and Methods. Nuclear fractions were isolated. Samples were run on a SDS-PAGE gel and probed with the indicated Abs (n=3). **B&D**. Human IFNβ was measured in cell supernatant from **A&C** by ELISA (n=3). **E&F**. *R232/R232, HAQ/HAQ, H232/H232 and HAQ/H232* human B cells from indicated ethnic groups were activated with CDG (10 μg/ml) for 5 h in culture. Nuclear fractions were isolated, run on a SDS-PAGE gel and probed with the indicated Abs (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.

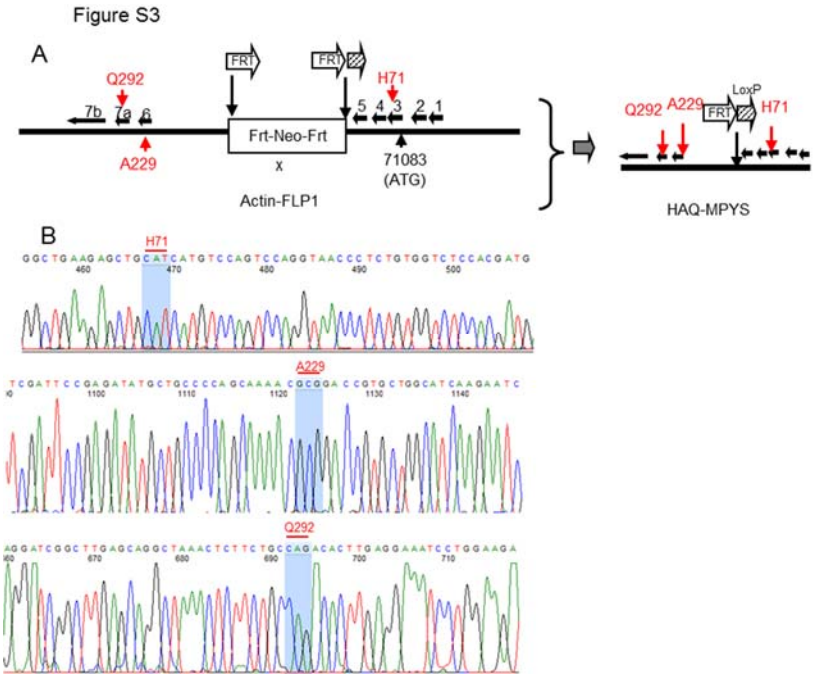

Figure S3. Making a HAQ knock-in mouse. **A.** Diagram illustrating the generation of an *HAQ* knock-in mouse. **B**. PCR sequencing of the H71-A229-Q292 mutations in the *HAQ* knock-in mouse.