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A Frequent Hypofunctional *IRAK2* Variant Is Associated With Reduced Spontaneous Hepatitis C Virus Clearance

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Patients carrying very rare loss-of-function mutations in interleukin-1 receptor–associated kinase 4 (*IRAK4*), a critical signaling mediator in Toll-like receptor signaling, are severely immunodeficient, highlighting the paramount role of IRAK kinases in innate immunity. We discovered a comparatively frequent coding variant of the enigmatic human *IRAK2*, L392V (rs3844283), which is found homozygously in ∼15% of Caucasians, to be associated with a reduced ability to induce interferon-alpha in primary human plasmacytoid dendritic cells in response to hepatitis C virus (HCV). Cytokine production in response to purified Toll-like receptor agonists was also impaired. Additionally, rs3844283 was epidemiologically associated with a chronic course of HCV infection in two independent HCV cohorts and emerged as an independent predictor of chronic HCV disease. Mechanistically, IRAK2 L392V showed intact binding to, but impaired ubiquitination of, tumor necrosis factor receptor–associated factor 6, a vital step in signal transduction. *Conclusion*: Our study highlights *IRAK2* and its genetic variants as critical factors and potentially novel biomarkers for human antiviral innate immunity. (HEPATOLOGY 2015;62:1375-1387)

The mammalian innate immune system relies on Toll-like receptors (TLRs) for the detection of invading microbes based on microbe-associated molecular patterns. For example, TLR2 detects bacterial lipoproteins, TLR4 detects lipopolysaccharide (LPS), and TLR7/8 detect bacterial and viral RNA.[1] TLR activation by microbe-associated molecular patterns triggers cytoplasmic signaling, culminating in the transcriptional

Abbreviations: DD, death domain; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; HCV, hepatitis C virus; HEK, human embryonic kidney; IFN, interferon; IL, interleukin; IL-1R, IL-1 receptor; IRAK, IL-1 receptor–associated kinase; KD, kinase domain; LPS, lipopolysaccharide; LUMIER, luminescence-based mammalian interactome mapping; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa light-chain-enhancer of activated B cells; OR, odds ratio; pDC, plasmacytoid dendritic cell; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, TNF-associated factor; WT, wild type.

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***See Supporting Information for the full names of all members of the East-German HCV Study Group and the Swiss Hepatitis C Cohort Study Group.*

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regulation of proinflammatory cytokines and interferons (IFNs), which initiate and shape adaptive immune responses.[1] The adaptor molecule myeloid differentiation primary response gene 88 (MyD88) plays a key role in integrating and diversifying signals elicited by all TLRs, except TLR3, and the interleukin (IL)−1 receptor (IL-1R), the mediator of the biological effects of the important inflammatory cytokine IL-1β.[2] Upon pathway activation, MyD88 associates and cooperates with kinases of the IL-1R-associated kinase (IRAK) family.[3] The pivotal importance of MyD88-IRAK4 signaling is evidenced by the fact that human carriers of rare *MYD88* or *IRAK4* loss-of-function mutations suffer from severe susceptibility to pyogenic bacterial infection.[4] Moreover, in various B-cell malignancies MyD88-IRAK signaling has been implicated in oncogenesis.[5,6] The IRAK family in humans and mice consists of four members each: IRAK1, IRAK2, IRAK3 (also termed IRAK-M). and IRAK4.[3] All IRAKs share an N-terminal death domain (DD), enabling interactions with the DD-containing MyD88 and a central kinase domain (KD). IRAK1-3 also feature a C-terminal extension, which contains motifs required for subsequent tumor necrosis factor receptor (TNF)–associated factor 6 (TRAF6) recruitment. MyD88–IRAK interactions take place in the context of the so-called Myddosome post–receptor complex, a hierarchical DD assembly of MyD88, IRAK4, and IRAK2.[7,8] Myddosome formation is initiated by MyD88 DD oligomerization into a ring-like structure that enables the recruitment of IRAK4 DD and IRAK2 DD, a process blocked by naturally occurring *MYD88* mutations.[7] The precise molecular events of signal transduction remain elusive; it is supposed that DD-mediated Myddosome formation enables proximity-induced activation of IRAK4 and IRAK1 or IRAK2 which, by an unknown mechanism, induces TRAF6 ubiquitination. This step is essential for the activation of nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase p38, and extracellular signal–regulated kinase.[1] Murine IRAKs have been well characterized using knockout and knockin models.[9,10] In one recent study in human peripheral blood mononuclear cells, IRAK2 was shown to be essential for the induction of TNF upon stimulation of TLR4 and TLR8, suggesting that IRAK2 may play an important role in TLR signaling in humans.[11] However, the respective roles and relative contributions of different IRAKs, apart from IRAK4, for immunity in humans remain surprisingly enigmatic to date. To elucidate the functional importance of IRAK2 in humans, we studied reported *IRAK2* genetic variants (nonsynonymous single-nucleotide polymorphisms [SNPs]) functionally in both model systems and human primary cells and epidemiologically with regard to viral disease. We discovered the frequent missense variant L392V as a hypofunctional *IRAK2* allele, showing a diminished ability to induce NF-κB signaling and cytokine induction in both cell lines and primary whole blood. In primary plasmacytoid dendritic cells (pDCs), IFN-α production in response to hepatitis C viral (HCV) infection was also compromised. Epidemiological association studies showed that the L392V (rs3844283) variant was associated with reduced spontaneous HCV clearance. The reduced functionality of L392V was found to stem from a failure to induce TRAF6 ubiquitination.

Materials and Methods

Reagents and Cell Lines. Reagents were from Sigma unless otherwise stated. For details on TLR ligands and antibodies, see Supporting Information. Human embryonic kidney (HEK) 293 cells were cultured as described.[7] Immortalized murine macrophages were previously generated by Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA) and cultured as described.[12]

Plasmids, Cloning, and Site-Directed Mutagenesis. Mutations corresponding to *IRAK2* nonsynonymous SNPs (Supporting Table S1) were introduced into human IRAK2 (Imagenes IRCMP5012D0935D) entry clones using the QuikChange XL Kit (Agilent). IRAK2, IRAK4, and TRAF6 entry clones were transferred into plasmids containing N-terminal *Renilla* luciferase or protein A tags,[7] N-terminal or C-terminal Strep-

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hemagglutinin (HA) tags (T. Bürckstümmer, CeMM, Vienna, Austria, and M. Gstaiger, ETH Zurich, Zurich, Switzerland), or N-terminal Flag tags (S. Pusch, Neuropathology, Heidelberg University, Heidelberg, Germany) by Gateway cloning (Life Technologies). For further details, see Supporting Information. Polymerase chain reaction and mutagenesis primers are listed in Supporting Table S4.

Retroviral Transduction and Functional Analysis of Immortalized* Irak2*-Deficient Macrophages. pMXs-IP-puro (MMLV LTR) (K.-M. Dennehy, Department of Virology, University of Tübingen, Tübingen, Germany), carrying either the empty MSC (mock), HA-tagged wild-type (WT), R214G, or L392V (introduced by conventional subcloning of respective polymerase chain reaction products), was used to transduce *Irak2*-deficient cells. Following puromycin selection (10 μg/mL), HA-IRAK2 expression was verified by anti-HA immunoblot. For TNF enzyme-linked immunosorbent assay (ELISA; Biolegend) experiments, macrophages were seeded in 96-well plates (2.5×10^4 cells/well) and stimulated with 1 μg/mL Pam_2CSK_4, 0.05 μg/mL LPS, 1 μg/mL R848, or 10 μg/mL poly(I:C) for 16 hours, and TNF in the supernatants was quantified by ELISA.

HEK293T Dual Luciferase Assays and ELISA. HEK293 cells were transfected with a firefly luciferase reporter (100 ng; Stratagene), pRL-Tk (10 ng; Promega), *Renilla* luciferase control reporter, pC1-EGFP (100 ng; Clontech), and IRAK2 plasmids (as indicated). Dual luciferase assays were performed 48 hours later,[7,13] and IL-8 in culture supernatants was determined by ELISA.

Coimmunoprecipitation and Expression Analysis. Immunoprecipitation experiments were done as described.[7] TRAF6 ubiquitination analysis was performed as described.[13]

Luminescence-based mammalian interactome mapping (LUMIER) was done as described recently[7]; for further details, see Supporting Information.

Isolation and Analysis of Whole Blood. See Supporting Fig. S5 for a work flow overview of analyses in primary blood. Heparinized whole blood was drawn from healthy volunteers at the Department of Immunology, Tübingen, upon written informed consent and according to a study protocol approved by the local ethics committee. None of the healthy volunteers indicated a history of any recurrent bacterial or viral infections. Further details on the characteristics of this study cohort are given in Supporting Table S6. For the analysis of cytokine release, whole blood was treated as indicated and incubated for 6 hours at 37°C and 5% CO_2. Subsequently, samples were centrifuged and plasma samples removed for Luminex analysis. For phosflow analysis whole blood was treated as indicated and incubated for 10 minutes at 37°C in a water bath following fixation and staining, as detailed in Supporting Information. A standardized protocol and identical flow cytometer (FacsCanto II; BD) settings were always used for all donors.

Luminex Cytokine Analysis. In Luminex analytes IL-1β, IL-6, MCP-1, IL-8, IP10, TNF, IL-1RA, IL-10, IL-13, IL-12p70, IL-1α, IL-5, and IFN-γ were quantified using in-house-developed bead-based sandwich immunoassay panels (IL-13, IL-12p70, and IFN-γ were not analyzed further as they were not induced in all donors). For further details regarding calibration, see Supporting Information. All donors were measured simultaneously using a Luminex FlexMAP 3D instrument. As the intention was to screen for cytokine responses affected between WT and homozygous groups, multiple testing was not corrected for; and asterisks denote comparisons that are nominally statistically significant ($P < 0.05$).

IFN Bioassays. IFN bioassays were performed by incubating plasma samples on HEK-Blue IFN-α/β cells (Invivogen), which contain an IFN-α/β inducible ISG54 promoter reporter for secreted embryonic alkaline phosphatase, which was quantified (QUANTI-Blue; Invivogen).

Isolation and HCV Infection of Primary pDCs. pDCs were isolated from buffy coats of healthy donors by Ficoll density centrifugation and magnetic cell sorting separation (Diamond Plasmacytoid Dendritic Cell Isolation Kit; Miltenyi). All donors provided written informed consent. Isolated pDCs were cultivated in serum-free dendritic cell medium (CellGenix), enriched with 10 ng/mL IL-3 (CellGenix), and then stimulated with a crude HCV Jc1 preparation of multiplicity of infection 3. After 18 hours, IFN-α was measured by ELISA (eBioscience). For further details, see Supporting Information.

Sequence Alignments, Structural Analysis, and Homology Modeling. Protein sequences from the National Center for Biotechnology Information were aligned with ClustalX. The homology model for the IRAK2 KD was generated using the MODELLER package[14] as described[15,16] based on the structure of the IRAK4 KD (pdb code 2NRU).[17]

Polymorphism Information and SNP Selection. A list of reported SNPs in human *IRAK2* (gene identification 4615) was obtained from the National Center for Biotechnology Information's dbSNP at www.ncbi.nlm.nih.gov/snp/(Supporting Table S1), and initially these coding SNPs were screened, irrespective of minor allele frequency. HapMap data were from www.hapmap.org.[18]

Genotyping. Genotyping in the German HCV cohort was performed by real-time polymerase chain

reaction and melting curve analysis (see Supporting Table S5 for primers/probes). Healthy donors were genotyped using a commercially available and prevalidated TaqMan assay (Life Technologies) for rs3844283. Polymorphisms in patients from the Swiss HCV cohort were extracted from a genome-wide association study–generated data set.[19]

Statistical Analysis. For functional experiments *P* values were determined using an unpaired Student *t* test, when comparing technical triplicates. Otherwise, a Mann-Whitney U test was used to account for a non-gaussian distribution. *P* values were calculated in GraphPad Prism 5.0 or Excel 2010 and considered significant at $P < 0.05$ (denoted by asterisk). The identity of statistical tests used in each experiment is given for each subpanel in the respective figure legends. Statistical analyses for epidemiological associations were performed with SPSS 20.0 (SPSS) or with SAS 9.2 (SAS Institute). The significance of differences was assessed in contingency tables using Pearson's χ^2 test and Fischer's exact test. Simple and stepwise multiple regression analyses adjusted for sex, age, and *IL28B* SNPs rs12979860 and rs8099917 were performed to determine factors associated with spontaneous clearance. The odds ratio (OR) and 95% confidence interval were calculated. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Human Subjects. For all experiments involving blood or purified cells from healthy volunteers, informed consent in writing was obtained from each volunteer; and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki as reflected in *a priori* approval by the appropriate local institutional review committees. The genotyping studies in the German and Swiss HCV cohorts were approved by the Ethics Committees of Medical Research of the University of Leipzig and from the eight Swiss participating hospitals' ethics committees, respectively, in accordance with the Declaration of Helsinki. All patients provided written informed consent. For further details, see Supporting Information.

Animal Experimentation. This study did not involve work with live animals or cells isolated from live animals; see details for immortalized murine macrophages in Supporting Information.

Results

The IRAK2 Variant L392V Is a Hypofunctional IRAK2 Allele. In order to study the functional importance of human IRAK2, we selected *IRAK2* germline variants reported in public databases with an estimated frequency of >0.1% in Caucasian populations (see Fig. 1A; Supporting Table S1). These variants were screened for their ability to activate NF-κB and induce IL-8 secretion in an overexpression assay in HEK293T cells,[20] using Strep- HA-tagged *IRAK2* point mutants equivalent to the reported variants. Several *IRAK2* variants showed significantly decreased (hypofunctional) NF-κB activation and IL-8 secretion compared to WT IRAK2 (Fig. 1B,D), despite comparable expression (Fig. 1C). The kinase domain variant L392V (corresponding to rs3844283) showed a moderate but consistent and significant reduction in NF-κB activation by ~30% (Fig. 1B) and ~20% reduced IL-8 secretion (Fig. 1D). As rs3844283 is highly frequent in different ethnicities (Fig. 1E), and thus highly amenable to epidemiological genetic studies, and leucine at position 392 is highly conserved (Supporting Fig. S1A), we decided to explore the L392V variant further. In line with the initial discovery of IRAK2 as part of the IL-1R pathway,[21] we also noted an effect of IRAK2 L392V overexpression on NF-κB activation in response to simultaneous IL-1β stimulation in HEK293T cells (Fig. 1F). Predictions of the structural and functional impact of the leucine to valine exchange suggested that the affected residue position maps in close proximity to the putative activation loop of IRAK2 (Supporting Fig. S1A,B), and it is predicted to have a potentially "damaging" effect (Supporting Table S2) using the analysis algorithms PolyPhen-2 and SIFT.[22,23]

We next sought to verify the putative hypofunctional phenotype of IRAK2 L392V in a more physiological system, allowing for endogenous TLR signal stimulation. Because TLR4 and TLR8 signaling in human peripheral blood mononuclear cells was reported to be IRAK2-dependent,[11] we stimulated whole blood from healthy volunteers homozygous for the WT allele (392LL) versus homozygous 392VV carriers with TLR3 (poly[I:C], considered IRAK2-independent; see Kawagoe et al.[24]), TLR4 (LPS), TLR7/8 ligand (R848), and IL-1R (IL-1β). In mice, Irak2 seems critical for late (later than 2 hours) signaling but redundant in initial signaling transduction.[10,24] In agreement with these findings and confirming a similar overall "fitness" of the assayed blood immune cells from 392LL versus 392VV carriers, no significant differences were observed in gated monocytes from 392LL (WT) versus 392VV carriers regarding early (10-minute time point) CD62L shedding and p38, extracellular signal–regulated kinase, or p65 phosphorylation (Supporting Fig. S2). Conversely, and despite expected donor-to-donor variation, after 6 hours of TLR stimulation, plasma cytokine protein levels were found to be significantly lower in 392VV

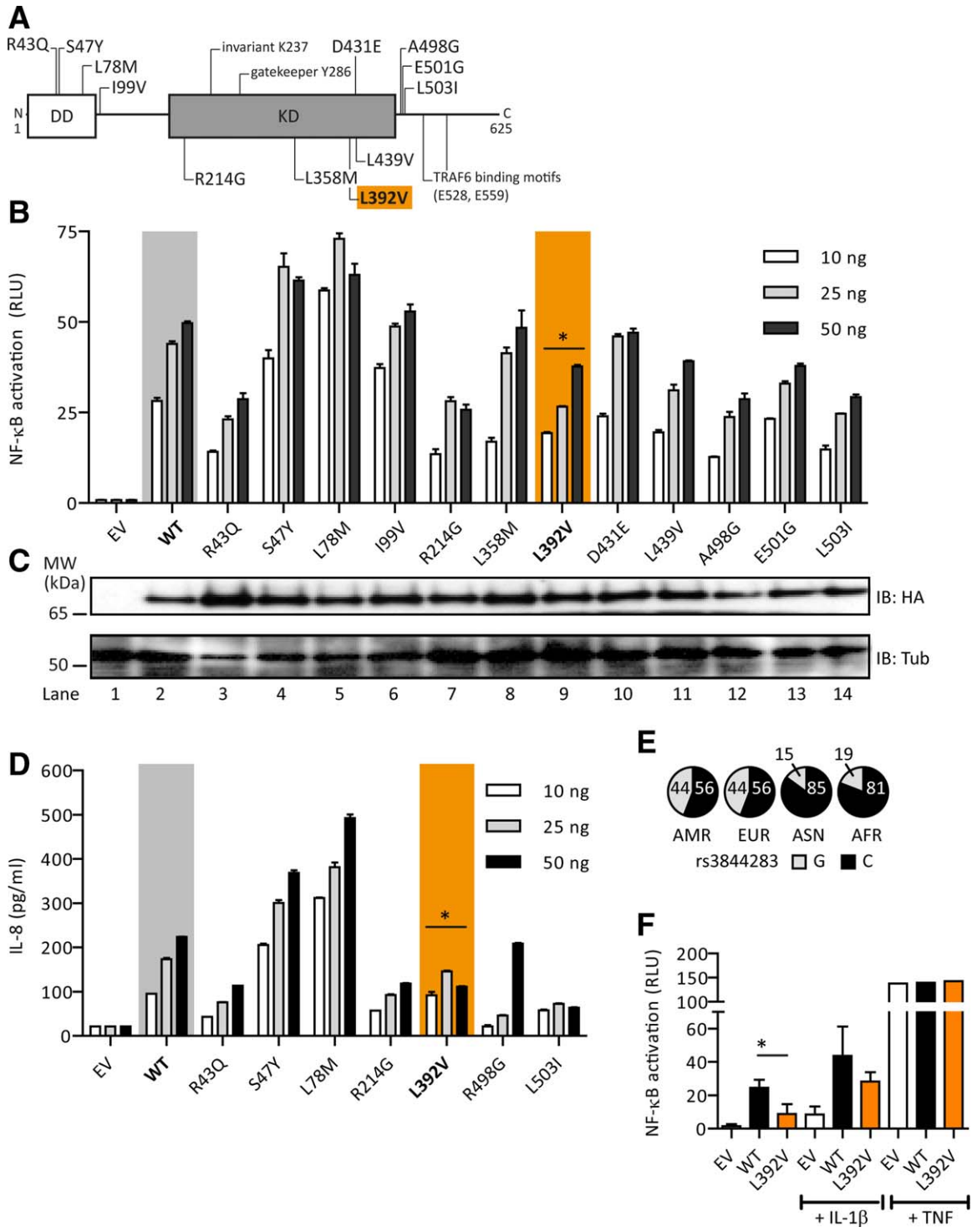

Fig. 1. IRAK2 L392V shows reduced NF-κB activation. (A) Schematic overview of human IRAK2 domains with nonsynonymous SNPs as indicated and L392V highlighted in orange. (B) IRAK2 L392V (shaded orange) shows reduced NF-κB induction (quantified by dual luciferase assay in triplicates $\pm$ standard deviation) compared to WT (shaded gray) in HEK293T cells transiently transfected with the indicated amounts of Strep-HA-tagged IRAK2 constructs. (C) Expression of IRAK2 variants is comparable to WT. HEK293T cells were transfected with Strep-HA-tagged expression constructs and analyzed by anti-HA immunoblot. (D) IL-8 secretion is reduced for L392V (shaded orange) compared to WT (shaded gray). In the same supernatants from HEK293T cells transiently transfected with IRAK2 variants in (B), secreted IL-8 levels were quantified by ELISA (triplicates $\pm$ standard deviation). (B-D) One representative out of at least three independent experiments is shown. In (B) and (D) *P* values using an unpaired *t* test are only shown for L392V for clarity. (E) IRAK2 rs3844283 is frequent in different ethnic groups according to phase 1 of the 1000 Genomes project. The G allele is the minor allele and codes for L392V. (F) IRAK2 L392V (orange) shows lower NF-κB induction (quantified by dual luciferase assay) compared to WT (black bars) in HEK293T cells transiently transfected with Strep-HA-tagged IRAK2 constructs upon stimulation with IL-1β. TNF-induced NF-κB activation was indistinguishable. Data were combined from three identical experiments, each done in triplicate. Means $\pm$ standard error of the mean are shown. In (B) and (D) an unpaired *t* test was used, and in (F) a Mann-Whitney U test was used. Abbreviations: AMR, American; AFR, African; ASN, Asian; EUR, European; EV, empty vector; RLU, relative light units; Tub, tubulin (loading control).

donors, when all serum samples were analyzed in parallel using a screening approach by bead-based multiplex cytokine analysis (Luminex). Especially IL-1β, MCP-1, and IL-8 protein levels were found to be nominally statistically significantly lower in 392VV minor allele carriers in response to the TLR7/8 ligand R848 (Fig. 2A); MCP-1, IL-8, and TNF levels were also significantly reduced in response to TLR4 LPS stimulation (Fig. 2B). Although some additional cytokines (e.g., IL-6; Supporting Fig. S3A) were nonsignificantly reduced in 392VV carriers, the levels of IL-1α, IL-5, IL-10, IL-1RA, or IP10 upon LPS or R848 stimulation were comparable between 392VV and 392LL carriers (not shown). IL-1β only significantly induced IL-8 with a trend toward lower IL-8 concentrations in 392VV carriers (Supporting Fig. S3B). The IRAK2-independent[24] (see Supporting Fig. S3B) TLR3 control stimulus poly(I:C) only (weakly) induced IP10 in this whole-blood assay but showed no significant differences between the two groups (Supporting Fig. S3C). Type 1 IFN levels were also determined by an IFN bioassay (HEK-Blue IFN-α/β cells, see Materials and Methods) in 392LL and 392VV carriers and showed a trend toward slightly lower IFN levels for LPS, but the difference was not statistically significant. For R848, which is often used as a surrogate marker for viral RNA, no difference was observed (Fig. 2C). To corroborate these findings from primary human samples, in a second, more easy-to-manipulate system, murine immortalized macrophages from *Irak2*-deficient mice were reconstituted to equal expression levels (Fig. 2D) with a retrovirus containing a puromycin-selectable HA-tagged human WT or L392V IRAK2 cassette.[12] This conferred responsiveness to TLR2, TLR4, and TLR7 stimulation in terms of TNF protein secretion (Fig. 2E). L392V reconstituted macrophages produced less TNF protein than WT IRAK2 macrophages in response to R848, LPS, and the TLR2 ligand Pam_2CSK_4 (Fig. 2F) but not the IRAK2-independent poly(I:C) TLR3 stimulus (Supporting Fig. S3D). Collectively, analysis in both human primary cells and a murine model system suggested that IRAK2 L392V rs3844283 is a hypofunctional *IRAK2* allele associated with impaired TLR-induced cytokine responses.

L392V Carriers Fail to Induce IFN-α in Primary pDCs and Are Predisposed to Chronic HCV Infection. Considering the only subtle effect of L392V on type 1 IFN but pronounced reduction in proinflammatory cytokines in healthy donors in response to synthetic TLR agonists such as R848, a suboptimal inducer of antiviral genes,[25] we wondered if IFN production might be affected more strongly upon infection with an intact pathogen. It has been shown that HCV RNA is sensed by TLR7[26] and that TLR7 signals depend on IRAK2.[11,24] pDCs are professional viral sentinel cells, which functionally express TLR7,[27,28] and respond to HCV stimulation with IFN-α production.[29] We therefore purified primary pDCs from 30 healthy 392LL or 392VV donors and stimulated them with HCV Jc1 (multiplicity of infection = 3). As evident from Fig. 3A, pDCs from 392VV donors produced a median level of ~100 pg/mL of IFN-α, i.e., more than 83% less than WT (392LL) donors (~600 pg/mL). Heterozygotes displayed a nonsignificant trend toward lower IFN-α (median ~500 pg/mL). Thus, the hypofunctional effect of L392V extended to a purified primary cell type involved in viral surveillance, when encountering infection with a viral pathogen. This prompted us to investigate whether rs3844283 was also epidemiologically associated with HCV disease in humans, which depends on IFN for clearance.[30] Analysis of rs3844283 allele frequencies (Supporting Table S3) in the well-characterized German HCV cohort ($n = 895$, Wiese et al.[31]) showed that the rs3844283 G allele (coding for L392V) was generally associated with a lower OR to spontaneously clear HCV infection: in a dominant model, the G allele of *IRAK2* rs3844283 significantly decreased the odds for spontaneous HCV clearance by 34% (OR = 0.66, $P = 0.005$; Table 1 and visualized in Fig. 3B). This was confirmed in an independent collective, the Swiss HCV cohort ($n = 1019$, Rauch et al.[19]), where the OR for spontaneous clearance was moderately lower, 0.49 ($P = 0.0015$; Table 2; Supporting Table S3), confirming a 34%-51% reduced ability of rs3844283 G allele carriers to spontaneously clear HCV infection and, thus, a virtually doubled risk to develop chronic disease (Fig. 3B). There was no association with clearance under treatment. A coding SNP in IFN-λ family member *IL28B*, rs12979860, has been shown to correlate with HCV clearance.[32] To investigate whether the effect of rs3844283 was biased by these predictors of spontaneous HCV clearance as well as sex or age, we conducted a multivariate regression analysis in both the German and Swiss HCV cohorts, which showed that the rs3844283 G allele predicted statistically significantly reduced spontaneous virus clearance, independent of *IL28B* SNP carriage, sex, or age. After adjusting for these parameters, rs3844283 remained significantly associated with lower spontaneous viral clearance and, thus, chronic disease. In the final models the ORs were 0.68 ($P = 0.015$; Table 1) in the German HCV cohort and 0.47 ($P = 0.001$; Table 2) in the Swiss HCV cohort. Collectively, the rs3844283 G allele (coding for L329V) therefore

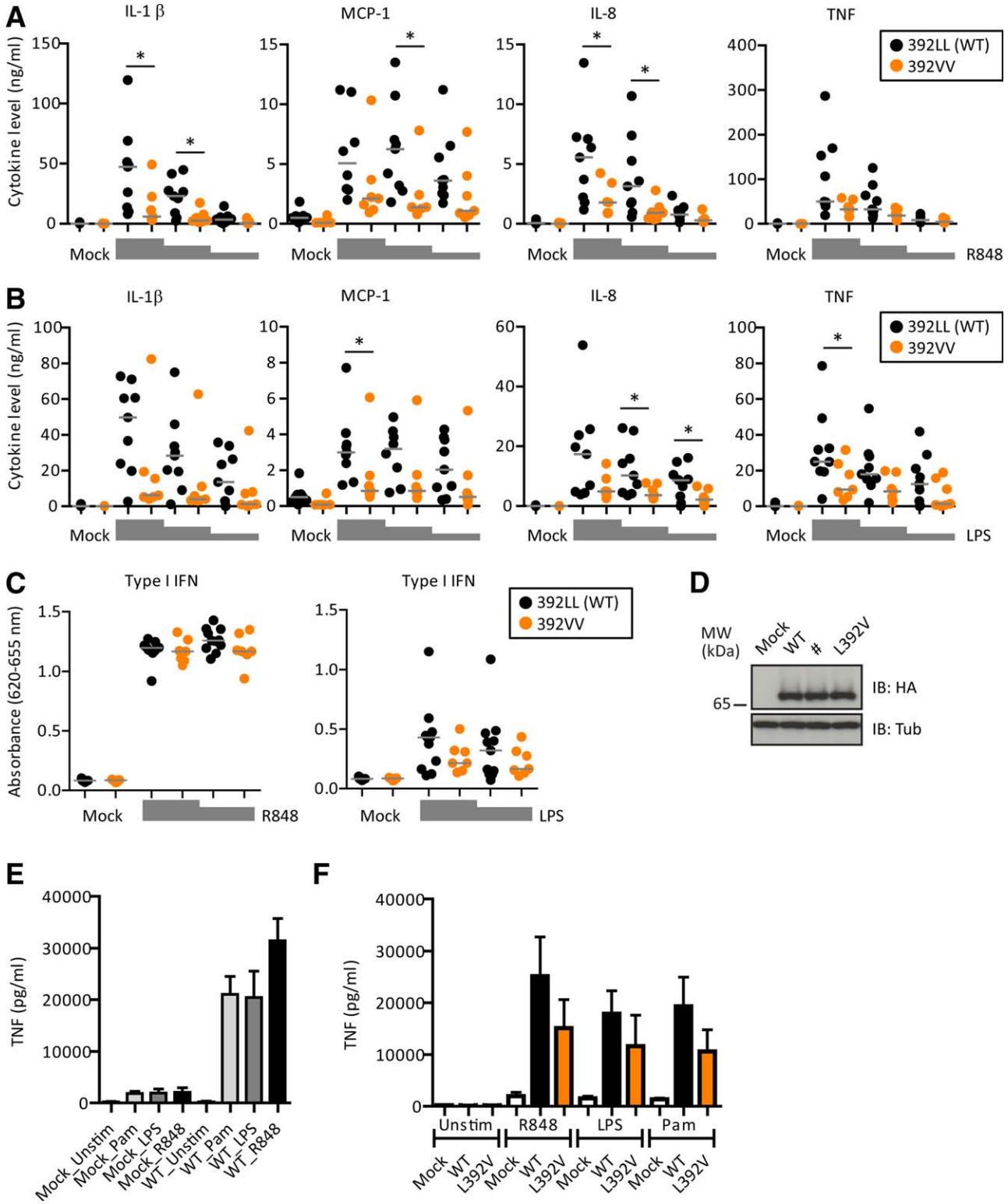

Fig. 2. Primary cells from IRAK2 L392V carriers and L392V-reconstituted murine macrophages show reduced cytokine responses upon stimulation with TLR ligands. (A-C) Whole blood from 392LL (black, n = 9) or 392VV (orange, n = 7) carriers was left untreated (Mock) or stimulated with either (A) R848 (5, 1, or 0.2 μg/mL) or (B) LPS (100, 10, or 1 ng/mL) for 6 hours, and cytokine levels in plasma were determined by Luminex bead arrays. Each dot represents one donor, medians in gray. All donors were measured in parallel. Due to the screening nature of the experiment, multiple testing was not corrected for, and *P* values are thus nominally significant. A Mann-Whitney U test was used. (C) as in (A) or (B) but using only the two highest concentrations of each ligand. Type 1 IFN levels were determined using HEK-Blue IFN-α/β cells (see Materials and Methods). (D) Anti-HA immunoblot analysis of whole-cell lysates of Mock, HA-IRAK2 WT, and HA-L392V reconstituted macrophages (see Materials and Methods). #Transduction with a construct irrelevant to this study. One out of three independent experiments shown. (E) Reconstitution of *Irak2*-deficient murine macrophages with human IRAK2 is functional as assessed by TNF secretion analyzed by ELISA (triplicates upon stimulation with 1 μg/mL Pam_2CSK_4 [light gray], 0.05 μg/mL LPS [dark gray], and 1 μg/mL R848 [black] for 16 hours). (F) TNF secretion in mock (white bars), WT human HA-IRAK2 (black), or L392V (orange) reconstituted macrophages upon stimulation with Pam_2CSK_4, LPS, or R848 for 16 hours. In (D) and (E) the means ± standard error of the mean of combined data of at least three independent experiments are shown. Abbreviations: IB, immunoblot; MW, molecular weight; Tub, tubulin (loading control).

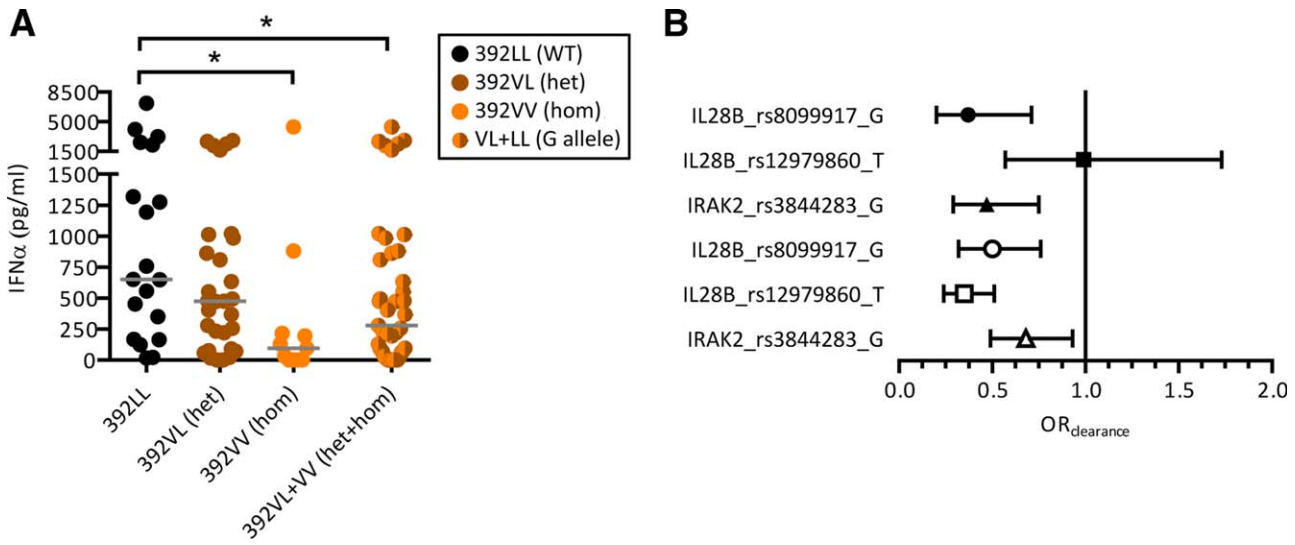

Fig. 3. IRAK2 rs3844183 G allele (L392V) carriers show a reduced IFN-α response in primary pDCs upon HCV infection and reduced spontaneous HCV clearance. (A) pDCs were isolated from 392LL (black, WT, n = 19), 392VL (ochre, heterozygotes, n = 30), or 392VV (orange, homozygote minor allele, n = 11) donors using magnetic beads; cultured; and infected with HCV Jc1 at multiplicity of infection = 3 for 18 hours and the released IFN-α was measured by ELISA. Each dot represents one donor, medians in gray. A Mann-Whitney U test was used to compare homozygous WT (black, LL) with heterozygotes (ochre, VL), homozygous minor allele (VV, orange), and all minor allele carriers (ochre/yellow semicircles, VL+LL). Jc1 (B) Graphic representation of the OR of *IRAK2* rs3844283 G allele (L392V; triangle), *IL28B* rs12979860 T allele (square), and *IL28B* rs8099917 G allele (circle), associated with spontaneous viral clearance in a multivariate stepwise logistic regression model in the German (open symbols) and Swiss (closed symbols) cohorts. Whiskers indicate the 95% confidence interval.

Table 1. Association of *IRAK2* rs3844283 and *IL28B* rs12979860 and rs8099917 With the Natural Course of HCV Infection (Spontaneous Clearance)

	Univariate Analysis		Multivariate Analysis*	
Variant and Genotypes	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
rs3844283 CC versus CG/GG	0.66 (0.49-0.88)	0.005	0.68 (0.49-0.93)	0.015
rs12979860 CC versus CT/TT	0.23 (0.17-0.31)	2.9×10^{-21}	0.35 (0.24-0.51)	5.2×10^{-8}
rs8099917 TT versus TG/GG	0.25 (0.18-0.35)	1.4×10^{-15}	0.50 (0.32-0.76)	0.001

*Adjustment for sex, age, and *IL28B* SNPs rs12979860 and rs8099917.
Abbreviation: CI, confidence interval.

emerges as a novel and independent indicator for the development of chronic HCV disease, in line with a defective ability of G allele carriers to mount cytokine (Fig. 2) and especially IFN-α (Fig. 3) responses.

L392V Fails to Induce TRAF6 Ubiquitination. In order to gain a first insight into the molecular basis for the hyporesponsiveness of the L392V allele, we analyzed its impact on IRAK2 function in postreceptor signaling. In this context, IRAK2 firstly completes Myddosome assembly by oligomerizing and docking onto a MyD88-IRAK4 platform,[7] secondly recruits TRAF6 through C-terminal binding motifs (Fig. 1A; Keating et al.[13]), and thirdly induces ubiquitination of TRAF6.[13] We therefore first assessed the different protein–protein interactions by LUMIER.[7] The avidity of a protein–protein interaction is measured by coexpressing two interacting proteins with

Table 2. Association of *IRAK2* rs3844283 and *IL28B* rs12979860 and rs8099917 With the Natural Course of HCV Infection in the Swiss HCV Cohort (Spontaneous Clearance)

	Univariate Analysis		Multivariate Analysis*	
Variant and Genotypes	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
rs3844283 CC versus CG/GG	0.49 (0.31-0.78)	0.0015	0.47 (0.29-0.75)	0.001
rs12979860 CC versus CT/TT	0.56 (0.36-0.89)	0.013	0.99 (0.57-1.73)	0.977
rs8099917 TT versus TG/GG	0.39 (0.23-0.66)	0.00042	0.37 (0.20-0.71)	0.003

*Adjustment for sex, age, and *IL28B* SNPs rs12979860 and rs8099917.
Abbreviation: CI, confidence interval.

protein A (bait) and *Renilla* luciferase (prey) tags and subsequently determining the level of specific binding of the *Renilla*-tagged partner upon protein A purification. IRAK2 oligomerization and IRAK4 interaction showed no apparent differences between WT and L392V, but luminescence intensity ratios were low so that the strength of interaction could not be assessed reliably (data not shown). IRAK2–TRAF6 binding was normal as analyzed by LUMIER (Supporting Fig. S4) and coimmunoprecipitation using Flag-tagged TRAF6 (Fig. 4A), suggesting that L392V also bound TRAF6 comparably to WT—congruent with the fact that the L392V amino acid exchange maps distally to the IRAK2 TRAF6-binding motifs. However, the levels of TRAF6 ligation with HA-tagged (Fig. 4B) or endogenous ubiquitin (Fig. 4C) conferred by the expression of WT IRAK2[13] were both strongly reduced for L392V. This was similar to two described control mutants of IRAK2, E528A[13] (see lanes 3 and 6 or 3 in Figs. 4B or C, respectively) and R214G,[33] which both retain TRAF6 binding but fail to induce its ubiquitination. In good agreement, a recently reported hyperactive IRAK2 mutant, rs708035, hyperubiquitinates TRAF6,[34] suggesting that signaling output initiated by IRAK2 and TRAF6 ubiquitination are closely linked. Thus, despite intact Myddosome interactions, L392V is unable to fulfill its role as an inducer of TRAF6 ubiquitination, which is a requirement for TLR-mediated cytokine and IFN production.[1] We propose that defective TRAF6 ubiquitination is the molecular cause for the observed hypofunctional phenotype of the L392V variant.

Discussion

In a dawning age of individualized approaches to human health problems, the need to unravel genetic determinants of host susceptibility to infectious agents has been widely recognized. Although striking examples of an association between genetic variation and host susceptibility—e.g., *MYD88* and *IRAK4* loss-of-function alleles[4]—have highlighted the contribution of TLR-MyD88-IRAK4 signaling to the human immune system, their applicability to diagnostic, therapeutic, or preventative exploitation is very limited due to their extremely low frequency (estimated <0.01%). More frequent variants *ab initio* are expected to contribute less to disease susceptibility in the general population but may, on the other hand, contribute with moderate ORs to higher or lower disease susceptibilities in thousands of individuals.[35] We here report such a frequent functional variant in *IRAK2* that occurs homozygously in ~15% (and thus millions) of Caucasians. Although two other *IRAK2* variants were reported by us and others to be functionally attenuated,[33,34] L392V is the first variant that is frequent and for which a disease association in multiple disease cohorts, a hypofunctional phenotype in human primary cells, and a molecular investigation have been described.

On the molecular level, L392V-associated hypoactivity seems to be due to reduced TRAF6 ubiquitination. From a mechanistic point of view, this is in agreement with earlier studies showing that the recruitment of TRAF6, e.g., through IRAK2 or Mal/TIRAP, is necessary but not sufficient to initiate downstream signaling.[10,13,36] It remains unclear whether IRAK2–TRAF6 association must occur prior to the recruitment of IRAK2 to active Myddosomes or subsequently. As aforementioned, the efficiency of TRAF6 ubiquitination seems to correlate with ultimate NF-κB activation and thus TLR pathway functionality. Because TRAF6 binding was intact and L392V does not map to the IRAK2 C terminus, L392V may allosterically influence the IRAK2 C-terminal domain harboring the TRAF6 recruitment motifs. Otherwise, the proximity of L392V to the putative IRAK2 activation loop (Supporting Fig. S1B,C) may result in a reduced ability to serve as a substrate for upstream phosphorylation, e.g., by IRAK4. It can only be speculated whether, alternatively, the KD variant L392V may affect IRAK2 catalytic activity. Because there is evidence both for[24] and against[37] a catalytic role of the IRAK2 KD, to solve the still enigmatic relationship of Myddosome assembly, catalytic events, and TRAF6 ubiquitination and the influence of L392V will require detailed mechanistic studies outside the scope of the present study. However, our data provide evidence regarding the relative contribution of IRAK1 versus IRAK2 in TLR signaling in human primary cells. Studies in mice suggest a sequential requirement of IRAK1 (early signaling) and IRAK2 (late signaling),[10,24] but this has not been addressed satisfactorily in humans. Whereas for a hypofunctional allele in the upstream *TLR5* receptor flagellin-induced early (CD62L shedding and p38 phosphorylation) and late (cytokine induction) responses were affected,[38] our current data suggest that for IRAK2 L392V late, but not early, responses (CD62L shedding and p38, extracellular signal–regulated kinase, and p65 phosphorylation 10 minutes poststimulation) appear to be negatively affected in 392VV versus 392LL carriers (Supporting Fig. S2). This recapitulates data from *Irak2* knockin mice[10] and argues for sequential IRAK1 > IRAK2 employment in human primary cells. Collectively, our data show that the influence of IRAK2 on TLR signaling is not only important in mice or human cell lines but may have an impact on cytokine and IFN production in human primary cells in the context of infection.

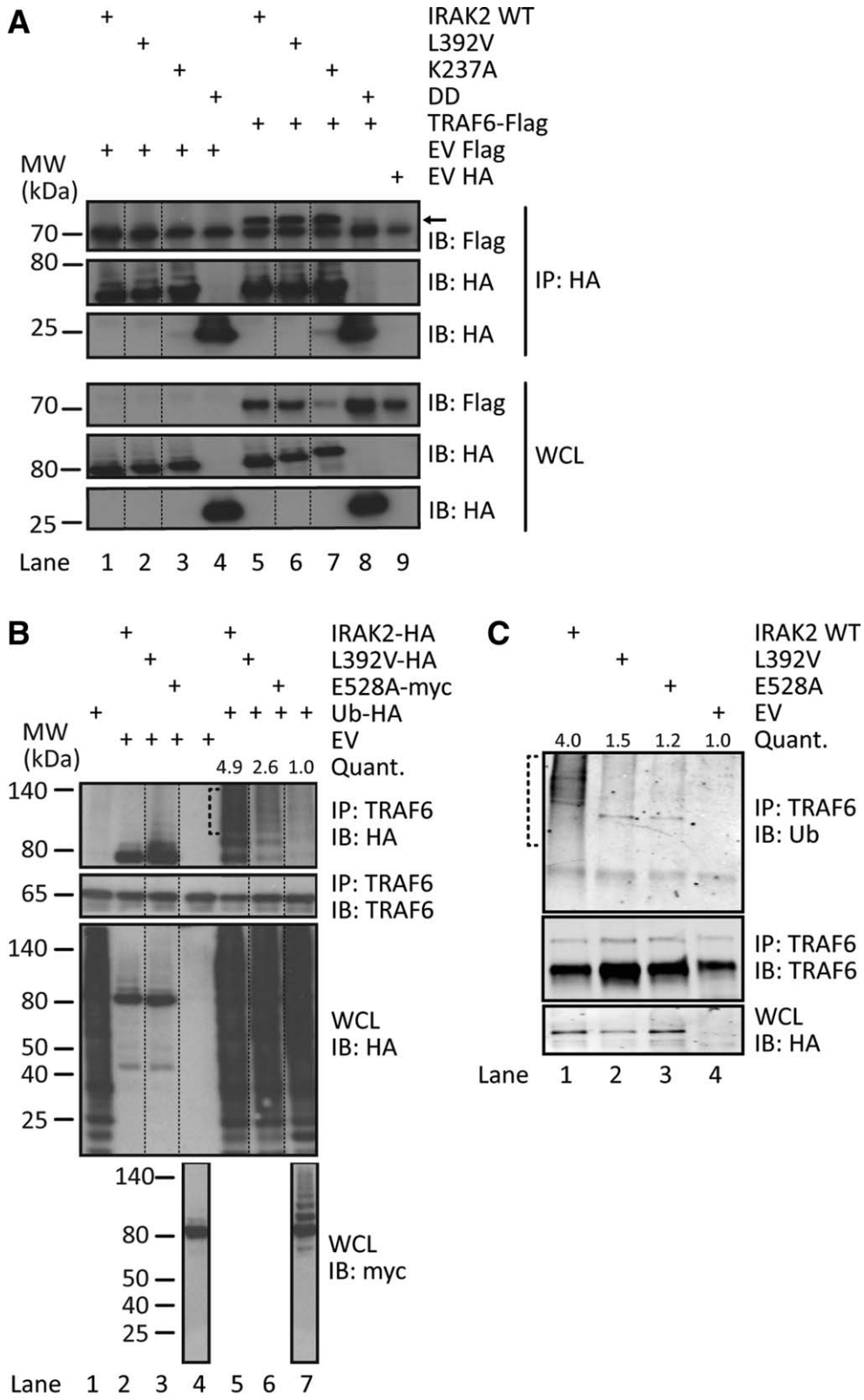

Fig. 4. IRAK2 L392V fails to induce TRAF6 ubiquitination. TRAF6 binding is comparable to WT as indicated by LUMIER luciferase analysis (Supporting Fig. S4) and coimmunoprecipitation (A) from HEK293T cells. (A) Upon expression of Strep-HA-tagged IRAK2 WT, variant, or DD-only constructs and Flag-TRAF6 in HEK293T cells, protein complexes were immunoprecipitated using anti-HA antibody. Precipitates and whole-cell lysates were analyzed by immunoblot as indicated. L392V coimmunoprecipitates with TRAF6 (arrow). (B,C) L392V fails to induce ubiquitination of TRAF6. (B) Upon expression of empty vector, Strep-HA-tagged IRAK2 WT or variant constructs, myc-IRAK2 E528A, and HA-ubiquitin in HEK293T cells, protein complexes were immunoprecipitated using anti-TRAF6 antibody. Precipitates and whole-cell lysates were analyzed by immunoblot as indicated. One representative out of five independent experiments is shown. (C) Analysis of endogenous ubiquitination of TRAF6. Upon expression of Strep-HA-tagged IRAK2 WT or variant constructs in HEK293T cells, protein complexes were immunoprecipitated using anti-TRAF6 antibody. Precipitates and whole-cell lysates were analyzed by immunoblot as indicated. High-molecular weight smears indicate TRAF6 ubiquitination. One representative out of four independent experiments is shown. Horizontal lines indicate where lanes irrelevant to this study were removed for improved clarity. Brackets indicate areas of ubiquitination quantification. Abbreviations: EV, empty vector; IB, immunoblot, IP, immunoprecipitation; MW, molecular weight; WCL, whole-cell lysate.

Indeed, our data implicate *IRAK2* variants in HCV pathogenesis in humans and suggest that IRAK2 may play an important role in human antiviral immunity. Although liver intrinsic mechanisms still appear to play a role,[39] recently published data suggest that immune surveillance of HCV critically depends on pDCs and other immune cells, which detect HCV based on viral RNA shed in exosomes from infected hepatocytes.[40,41] We therefore consider the defect in signaling and IFN production associated with IRAK2 L392V to be most relevant in immune cells such as pDCs. Apart from sensing through the TLR7–IRAK axis, retinoic acid –inducible gene–like helicases have been implicated in HCV RNA sensing.[42] Our results confirm the involvement of these pattern recognition receptor families on an epidemiological genetic basis. In line with the well-documented requirement for cytokines and IFNs in fighting viral infections,[43] carriage of the L392V minor allele was associated with ∼30%-50%, depending on the cohort, lower odds to spontaneously clear infections with HCV. Clearance under therapy was not associated here (data not shown). Association of IRAK2 rs3844283 with spontaneous clearance but not clearance under IFN-based therapy is in line with the notion that IRAK2 acts upstream of IFN signaling and IFN-stimulated gene regulation,[1,3] whereas some *IL28B* genotypes, which are associated with both, also appear to influence the latter.[44-48] On average, about 10%-20% of infected individuals are able to clear HCV infection spontaneously.[49] The remainder follows a chronic course of infection, thus facing long-term morbidities such as hepatocellular carcinoma or liver cirrhosis.[50] Until now, few underlying genetic factors for successful spontaneous clearance have been identified. Genetic variants in the type 3 IFN member *IL28B* (also referred to as IFN-*λ3*) were shown to be strong predictors of HCV chronification and/or response to therapy by us and others.[19,32,51-54] *IL28B* SNPs have therefore rapidly become useful diagnostic tools in individualized approaches to the treatment of HCV patients.[55] Our data suggest that rs3844283 (L392V) is rather a predictor of a chronic course of disease in HCV infection, and thus, rs3844283 (L392V) may aid in identifying individuals for which a "watch-and-wait" approach may be detrimental as they are highly unlikely to spontaneously clear the infection. In combination with *IL28B* genotyping, it could theoretically be envisaged that these individuals could be directly selected for treatment with the effective but currently highly expensive non-IFN HCV NS5B polymerase inhibitor sofobusvir,[56] for example. It will be interesting to investigate whether *IRAK2* alleles are associated with infections with other viral or bacterial agents that involve, for example, TLR7/8 recognition, such as Flaviviridae or group B streptococci.[57,58] Remarkably, *IRAK4* and *MYD88* loss-of-function alleles appear not to affect susceptibility to viral infection,[4] but according to our data, IRAK2 may have relevance for immune responses that involve type I IFN production in a chronic setting. Given that initial TLR signaling is intact (Supporting Fig. S2), it is conceivable that L392V is of functional importance only when sustained TLR responses are required for pathogen eradication in a chronic infection setting so that the course of an infection, rather than its initial manifestation, may be affected. Although carriage of alleles such as L392V does not seem to result in life-threatening infections *per se* (see Materials and Methods), in particular vulnerable individuals, such as young children, the elderly, and individuals undergoing immunosuppression, genotyping the rs3844283 *IRAK2* allele may be a useful tool for identifying high-risk individuals. On the other hand, IRAK2 being important for sustained TLR (and possibly IL-1R signaling; Fig. 1F; Supporting Fig. S3B) introduces the possibility that targeting IRAK2 in human patients may efficiently block inflammatory feed-forward loops by interfering with sustained TLR/IL-1R downstream signaling. Due to the redundancy with IRAK1 for immediate TLR responses, IRAK2 may emerge as a safer target than, for example, IRAK4 as targeting IRAK2 would leave some immediate, infection-relevant TLR responses intact.[10] Further studies need to be performed to investigate its ability to hydrolyze or at least bind adenosine triphosphate, which could be exploited to generate potent small molecule inhibitors.

In conclusion, we describe a frequent *IRAK2* variant, L392V, as a hypofunctional allele associated with chronic viral infection and attenuated cytokine and IFN production. Our study sheds new light on the so far enigmatic IRAK2 and identifies rs3844283 as a potential genetic IRAK2 biomarker. This warrants the further study of IRAK2 and rs3844283 in other chronic diseases with TLR/IFN involvement.

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References

1. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011;34:637-650.
2. O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. Immunol Rev 2008;226:10-18.

3. Flannery S, Bowie AG. The interleukin-1 receptor-associated kinases: critical regulators of innate immune signalling. Biochem Pharmacol 2010;80:1981-1991.
4. **Picard C**, **von Bernuth H**, Ghandil P, Chrabieh M, Levy O, Arkwright PD, et al. Clinical features and outcome of patients with IRAK-4 and MyD88 deficiency. Medicine 2010;89:403-425.
5. **Ngo VN**, **Young RM**, **Schmitz R**, **Jhavar S**, **Xiao W**, **Lim KH**, et al. Oncogenically active MYD88 mutations in human lymphoma. Nature 2011;470:115-119.
6. **Avbelj M**, **Wolz OO**, Fekonja O, Bencina M, Repic M, Mavri J, et al. Activation of lymphoma-associated MyD88 mutations via allostery-induced TIR domain oligomerization. Blood 2014;124:3896-3904.
7. George J, Motshwene PG, Wang H, Kubarenko AV, Rautanen A, Mills TC, et al. Two human MYD88 variants, S34Y and R98C, interfere with MyD88-IRAK4-myddosome assembly. J Biol Chem 2011;286: 1341-1353.
8. Gay NJ, Gangloff M, O'Neill LA. What the Myddosome structure tells us about the initiation of innate immunity. Trends Immunol 2011;32: 104-109.
9. **Yin W**, **Wan Y**, Kim TW, Yao P, Xiao H, Zhou H, et al. The kinase activity of interleukin-1 receptor–associated kinase 2 is essential for lipopolysaccharide-mediated cytokine and chemokine mRNA stability and translation. J Interferon Cytokine Res 2011;31:415-422.
10. **Pauls E**, **Nanda SK**, Smith H, Toth R, Arthur JS, Cohen P. Two phases of inflammatory mediator production defined by the study of IRAK2 and IRAK1 knock-in mice. J Immunol 2013;191:2717-2730.
11. Flannery SM, Keating SE, Szymak J, Bowie AG. Human interleukin-1 receptor–associated kinase-2 is essential for Toll-like receptor–mediated transcriptional and post-transcriptional regulation of tumor necrosis factor alpha. J Biol Chem 2011;286:23688-23697.
12. Nagpal K, Plantinga TS, Sirois CM, Monks BG, Latz E, Netea MG, et al. Natural loss-of-function mutation of myeloid differentiation protein 88 disrupts its ability to form Myddosomes. J Biol Chem 2011; 286:11875-11882.
13. Keating SE, Maloney GM, Moran EM, Bowie AG. IRAK-2 participates in multiple toll-like receptor signaling pathways to NFkappaB via activation of TRAF6 ubiquitination. J Biol Chem 2007;282:33435-33443.
14. Sali A, Overington JP. Derivation of rules for comparative protein modeling from a database of protein structure alignments. Protein Sci 1994;3:1582-1596.
15. Kubarenko A, Frank M, Weber AN. Structure–function relationships of Toll-like receptor domains through homology modelling and molecular dynamics. Biochem Soc Trans 2007;35:1515-1518.
16. Kubarenko AV, Ranjan S, Colak E, George J, Frank M, Weber AN. Comprehensive modeling and functional analysis of Toll-like receptor ligand-recognition domains. Protein Sci 2010;19:558-569.
17. Wang Z, Liu J, Sudom A, Ayres M, Li S, Wesche H, et al. Crystal structures of IRAK-4 kinase in complex with inhibitors: a serine/threonine kinase with tyrosine as a gatekeeper. Structure 2006;14:1835-1844.
18. International HapMap Consortium. The International HapMap Project. Nature 2003;426:789-796.
19. **Rauch A**, **Kutalik Z**, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 2010;138:1338-1345.
20. Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science 1997;278:1612-1615.
21. Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, et al. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J Biol Chem 1999;274:7611-7614.
22. **Adzhubei IA**, **Schmidt S**, **Peshkin L**, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248-249.
23. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4:1073-1081.
24. Kawagoe T, Sato S, Matsushita K, Kato H, Matsui K, Kumagai Y, et al. Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. Nat Immunol 2008;9:684-691.
25. Colak E, Leslie A, Zausmer K, Khatamzas E, Kubarenko AV, Pichulik T, et al. RNA and imidazoquinolines are sensed by distinct TLR7/8 ectodomain sites resulting in functionally disparate signaling events. J Immunol 2014;192:5963-5973.
26. Zhang YL, Guo YJ, Bin L, Sun SH. Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7. J Hepatol 2009;51:29-38.
27. Ito T, Wang YH, Liu YJ. Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9. Springer Semin Immunopathol 2005;26:221-229.
28. **Gilliet M**, **Cao W**, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 2008;8:594-606.
29. Grabski E, Wappler I, Pfaender S, Steinmann E, Haid S, Dzionek A, et al. Efficient virus assembly, but not infectivity, determines the magnitude of hepatitis C virus induced interferon-alpha responses of plasmacytoid dendritic cells. J Virol 2015;89:3200-3208.
30. Heim MH. Innate immunity and HCV. J Hepatol 2013;58:564-574.
31. Wiese M, Berr F, Lafrenz M, Porst H, Oesen U. Low frequency of cirrhosis in a hepatitis C (genotype 1b) single-source outbreak in germany: a 20-year multicenter study. HEPATOLOGY 2000;32:91-96.
32. **Thomas DL**, **Thio CL**, **Martin MP**, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
33. Wang H, Flannery SM, Dickhofer S, Huhn S, George J, Kubarenko AV, et al. A coding IRAK2 protein variant compromises Toll-like receptor (TLR) signaling and is associated with colorectal cancer survival. J Biol Chem 2014;289:23123-23131.
34. **Zhang W**, **He T**, Wang Q, Li X, Wei J, Hou X, et al. Interleukin-1 receptor–associated kinase-2 genetic variant rs708035 increases NF-kappaB activity through promoting TRAF6 ubiquitination. J Biol Chem 2014;289:12507-12519.
35. Schork NJ, Murray SS, Frazer KA, Topol EJ. Common versus rare allele hypotheses for complex diseases. Curr Opin Genet Dev 2009;19:212-219.
36. Mansell A, Brint E, Gould JA, O'Neill LA, Hertzog PJ. Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)−6 to mediate NF-kappaB activation by toll-like receptor (TLR)−2 and TLR4. J Biol Chem 2004;279:37227-37230.
37. Wesche H, Gao X, Li X, Kirschning CJ, Stark GR, Cao Z. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. J Biol Chem 1999;274:19403-19410.
38. **Klimosch SN**, **Forsti A**, Eckert J, Knezevic J, Bevier M, von Schonfels W, et al. Functional TLR5 genetic variants affect human colorectal cancer survival. Cancer Res 2013;73:7232-7242.
39. Wang Y, Li J, Wang X, Sang M, Ho W. Hepatic stellate cells, liver innate immunity, and hepatitis C virus. J Gastroenterol Hepatol 2013; 28(Suppl. 1):112-115.
40. Cosset FL, Dreux M. HCV transmission by hepatic exosomes establishes a productive infection. J Hepatol 2014;60:674-675.
41. Dreux M, Garaigorta U, Boyd B, Decembre E, Chung J, Whitten-Bauer C, et al. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. Cell Host Microbe 2012;12:558-570.
42. **Hoffmann F**, **Schmidt A**, Dittmann Chevillotte M, Wisskirchen C, Hellmuth JC, Willms S, et al. Polymorphisms in MDA-5 link protein function to clearance of hepatitis C virus. HEPATOLOGY 2015;61:460-470.
43. Howell J, Angus P, Gow P, Visvanathan K. Toll-like receptors in hepatitis C infection: implications for pathogenesis and treatment. J Gastroenterol Hepatol 2013;28:766-776.
44. Abe H, Hayes CN, Ochi H, Maekawa T, Tsuge M, Miki D, et al. IL28 variation affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy. J Hepatol 2011;54:1094-1101.

45. Raglow Z, Thoma-Perry C, Gilroy R, Wan YJ. IL28B genotype and the expression of ISGs in normal liver. Liver Int 2013;33:991-998.
46. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 2010;139:499-509.
47. **Urban TJ**, **Thompson AJ**, **Bradrick SS**, Fellay J, Schuppan D, Cronin KD, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. Hepatology 2010;52:1888-1896.
48. Smith KR, Suppiah V, O'Connor K, Berg T, Weltman M, Abate ML, et al. Identification of improved IL28B SNPs and haplotypes for prediction of drug response in treatment of hepatitis C using massively parallel sequencing in a cross-sectional European cohort. Genome Med 2011;3:57.
49. Zeuzem S, Berg T, Moeller B, Hinrichsen H, Mauss S, Wedemeyer H, et al. Expert opinion on the treatment of patients with chronic hepatitis C. J Viral Hepatitis 2009;16:75-90.
50. Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol 2006;45: 529-538.
51. Fischer J, Bohm S, Scholz M, Muller T, Witt H, George J, et al. Combined effects of different interleukin-28B gene variants on the outcome of dual combination therapy in chronic hepatitis C virus type 1 infection. Hepatology 2012;55:1700-1710.
52. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.
53. **Ge D**, **Fellay J**, **Thompson AJ**, **Simon JS**, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment–induced viral clearance. Nature 2009;461:399-401.
54. **Tanaka Y**, **Nishida N**, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
55. Matsuura K, Watanabe T, Tanaka Y. Role of IL28B for chronic hepatitis C treatment toward personalized medicine. J Gastroenterol Hepatol 2014;29:241-249.
56. Cholongitas E, Papatheodoridis GV. Sofosbuvir: a novel oral agent for chronic hepatitis C. Ann Gastroenterol 2014;27:331-337.
57. Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, et al. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol 2009;10:587-594.
58. Prasad L, Spicher VM, Zwahlen M, Rickenbach M, Helbling B, Negro F. Cohort profile: The Swiss Hepatitis C Cohort Study (SCCS). Int J Epidemiol 2007;36:731-737.

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