



HLA-H*02:07 Is a Membrane-Bound Ligand of Denisovan Origin That Protects against Lysis by Activated Immune Effectors

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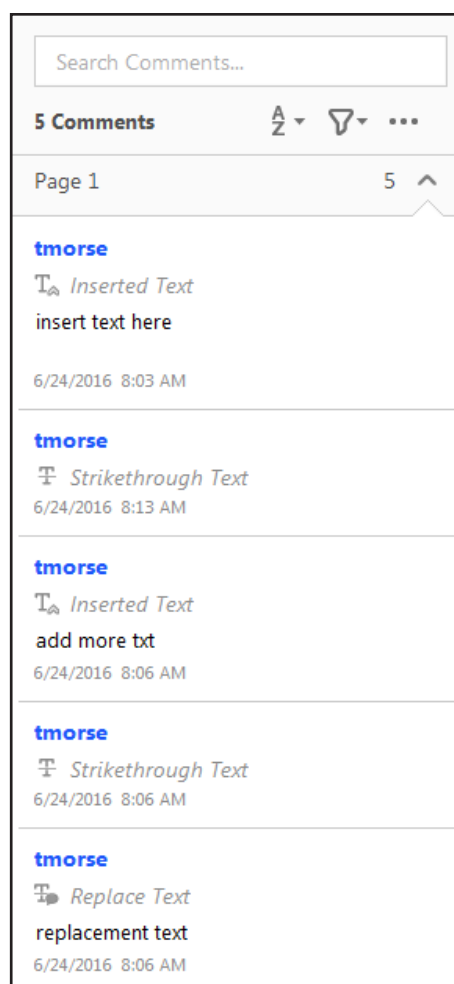
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HLA-H*02:07 Is a Membrane-Bound Ligand of Denisovan Origin That Protects against Lysis by Activated Immune Effectors

Lucas Hubert,^{*,†} Julien Paganini,[‡] Christophe Picard,^{*,†} Jacques Chiaroni,^{*,†}
Laurent Abi-Rached,^{§,¶} Pierre Pontarotti,^{§,¶} and Julie Di Cristofaro^{*,†}

The biological relevance of genes initially categorized as “pseudogenes” is slowly emerging, notably in innate immunity. In the HLA region on chromosome 6, *HLA-H* is one such pseudogene; yet, it is transcribed, and its variation is associated with immune properties. Furthermore, two *HLA-H* alleles, *H*02:07* and *H*02:14*, putatively encode a complete, membrane-bound HLA protein. Here we thus hypothesized that HLA-H contributes to immune homeostasis similarly to tolerogenic molecules HLA-G, -E, and -F. We tested if *HLA-H*02:07* encodes a membrane-bound protein that can inhibit the cytotoxicity of effector cells. We used an HLA-null human myeloid cell line transduced with *HLA-H*02:07* cDNA to demonstrate that *HLA-H*02:07* encodes a membrane-bound protein. Additionally, using a cytotoxicity assay, our results support that K562 *HLA-H*02:07* inhibits human effector IL-2-activated PBMCs and human IL-2-independent NK92-MI cell line activity. Finally, through in silico genotyping of the Denisovan genome and haplotypic association with Denisovan-derived *HLA-A*11*, we also show that *HLA-H*02:07* is of archaic origin. Hence, admixture with archaic humans brought a functional *HLA-H* allele into modern European and Asian populations. *The Journal of Immunology*, 2021, 208: 1–6.

The HLA region is the most polymorphic region of the human genome, displaying a breadth of functional diversity, in particular for the HLA proteins themselves thanks to their extreme allelic diversity. This region also encompasses many pseudogenes (1–3) (i.e., genes considered as defective and that are often derived from functional genes through processes such as retrotransposition of processed mRNAs or segmental duplications). Although both processes are associated with genome plasticity, their typical product, pseudogenes, is generally considered nonfunctional. However, an increasing number of studies identify functional processes attributable to pseudogenes, and direct investigations reveal biological roles, notably in innate immunity (4). Yet, the potential expression and function of the pseudogenes described in the HLA region are largely unexplored.

HLA-H was defined as a nonfunctional gene, notably because of its genetic deletion in a significant fraction of the population (5–8). The 50-kb deletion encompassing *HLA-H* is observed at a frequency of >10% in all continents and reaches 21% in East Asia (9).

Currently, 19 *HLA-H* alleles with a distinct sequence in the exons are described (10). These alleles display open reading frames ranging from 18 to 362 aa. All potential HLA-H allotypes lack the cysteine at codon 164 reported to be critical for the disulfide bond of the $\alpha 2$ domain; this feature was put forward to classify HLA-H as

nonfunctional (3). Of these 19 alleles, 2 (*HLA-H*02:07* and *H*02:14*) putatively encode a complete, membrane-bound HLA protein (9). Interestingly, early data on the distribution of *H*02:07* in modern populations and high linkage disequilibrium (LD) with *HLA-A*11* are both consistent with *H*02:07* having been introduced into modern human populations on one of the HLA haplotypes that are of Denisovan origin (11).

*HLA-H*02:07* and *H*02:14* alleles and putative products are highly related to the HLA-G molecule (9). HLA-G is a nonclassical HLA class I molecule (HLA-Ib; a group that also includes HLA-E and -F) that is well described for its tolerogenic activity in a clinical context. Indeed, HLA-G ligand modulates NK cell and CTL-mediated activity as well as B-lymphocyte proliferation through its interaction with the inhibitory receptors LILRB1, LILRB2, and KIR2DL4 (reviewed in [12]).

HLA-H transcriptional activity was characterized in PBMCs and human bronchial epithelial cells (13–16). HLA-H immune properties were also supported by different observations. An indirect role in HLA-E expression was shown, for example, because HLA-H's signal peptide mobilizes in vitro HLA-E to the cell surface (16), similarly to what is observed for other HLA molecules as well as for stress and virus peptides (17, 18). HLA-H absence may also impair immune-tolerance equilibrium: In lung transplant patients,

*Aix-Marseille University, l'Établissement Français du Sang, CNRS, Anthropologie Bio-Culturelle, Droit, Éthique et Santé, Biologie des Groupes Sanguins, Marseille, France; [†]Établissement Français du Sang, Provence-Alpes Côte-d'Azur et, Marseille, France; [‡]Xegen, Gemenos, France; [§]Aix Marseille University, Institut de Recherche pour le Développement, Assistance Publique Hôpitaux de Marseille, Microbes, Evolution, Phylogénie et Infection, Institut Hospitalier Universitaire-Méditerranée Infection, Marseille, France; and [¶]CNRS, Marseille, France

Q:11 ORCIDs: 0000-0001-7110-5465 (L.H.); 0000-0003-2369-9320 (J.P.); 0000-0001-9118-5239 (C.P.); 0000-0001-7202-3648 (P.P.); 0000-0001-7867-6455 (J.D.C.).

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Address correspondence and reprint requests to Dr. Julie Di Cristofaro, Aix-Marseille University, CNRS, Établissement Français du Sang, Anthropologie Bio-Culturelle, Droit, Éthique et Santé, Biologie des Groupes Sanguins, 13005 Marseille, France. E-mail address: julie.dicristofaro@efs.sante.fr

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Abbreviations used in this article: Ct, cycle threshold; LD, linkage disequilibrium; MFI, mean fluorescence intensity.

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the *HLA-G*01:04* allele, in LD with the *HLA-H* deletion, was associated with de novo donor-specific Ag (19). The potential tolerogenic function of the H*02:07 protein is supported by its association, through its full LD with the *HLA-A*11* allele, with higher risk of lung cancer (15).

We hypothesize that HLA-H contributes to immune homeostasis similarly to tolerogenic molecules HLA-G, -E, and -F (20–26) and that *HLA-H*02:07* is expressed as a functional, immune-tolerant, membrane-bound HLA molecule. Here we thus aimed to study membrane expression of *HLA-H*02:07* in an HLA-null cell line and its potential function as an immune modulator in a cytotoxicity assay. HLA-G, which protects against NK cell cytotoxicity (27), was used as positive control. We also investigated, through the study of the Denisovan  locus, the possibility that *HLA-H*02:07* is of Denisovan origin and was introduced into modern populations in Asia by Denisovan admixture.

Materials and Methods

HLA-H and HLA-G K562 transduction

 *H*02:07* membrane expression was explored by transduction of *HLA-H*02:07* cDNA in the K562 human erythroleukemic cell line that displays a reduced expression of HLA class I. *HLA-G*01:01* cDNA transduction was used as a positive control. The K562 (ACC86) cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Braunschweig, Germany). *HLA-H*02:07* and *HLA-G*01:01* cDNAs optimized for protein expression (Gene Optimizer Assisted; Invitrogen) were cloned in the pWPXL expression vector (Addgene).

K562 cells were transduced with lentiviral particles. Lentiviral particles were harvested at days 1 and 2 after transfection of 25×10^6 HEK adherent cells at 60% of confluence with 12.5 μ g of pMDG (envelope vector), 25 μ g of p8.91 (packaging vector), and 50 μ g of pWPXL containing cDNA, 100 mM NaCl, and 1 mM polyethylenimine (Sigma-Aldrich). Lentiviral particles were concentrated by centrifugation at 9700 Rpm for 1 h 30 min in polyethylene glycol 10% (PEG 8000; Fluka Biochemika). K562 were transduced with lentiviral particles containing *HLA-H*02:07* or *HLA-G*01:01* and expanded.

Transcriptional expression of  *H* and  *G* transgenes in K562 cells was assessed by quantitative PCR. Total RNA from transduced K562 cells and wild-type K562 cells (negative control) was isolated using the RNeasy kit (Qiagen, Courtaboeuf, France). cDNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen).

Real-time PCR analyses were performed with lentivirus-specific transgene (*WPRL* gene) primers, *Albumin* gene primers were used as a reference (28). A primer specific to *HLA-H* cDNA was also included in the analysis (Table I).

Each experiment was carried out in duplicate, and average cycle threshold (Ct) was calculated with StepOne 2.1 software (Invitrogen), excluding Ct duplicates with an SD >0.5.

HLA-H and HLA-G membrane expression

HLA-H protein expression at the cell surface was assessed by flow cytometry. HLA-G expression was used as a positive control; wild-type K562 cells were used as a negative control. HLA-H and HLA-G membrane-bound proteins were stained by mouse IgG1 Ab clone W6/32-PE (anti-HLA class I; Invitrogen) and mouse IgG1 Ab clone B2M-01-PE (anti β_2 -microglobulin; Invitrogen). The isotype control used was mouse IgG1 Ab clone 679.1Mc7-PE (Beckman Coulter).

Data were acquired on a Cytotflex machine (Beckman Coulter) and analyzed with CytExpert software 2.3 and Kaluza Analysis 2.1. Membrane expression was estimated by mean fluorescence intensity (MFI).

HLA-H immunomodulation analysis

The capacity of membrane-bound HLA-H to inhibit the cytotoxic activity of immune effector cells was assessed by a cytotoxicity assay on target cells. Transduced and wild-type K562 cells were used as target cells, and IL-2-activated PBMCs or the NK92-MI cell line (an IL-2-independent NK cell line derived from the NK-92 cell line) were as effector cells.

The K562 cells display a reduced expression of HLA ligand for inhibitory NK receptors and high expression of ligands for activating NK receptors. Thus, activated immune effector cells exert a cell-mediated cytotoxic activity on K562 cells (target), which can be suppressed by engagement of inhibitory receptors expressed on the immune effectors (29). HLA-H*02:07 K562-transduced cells were used to explore

the capacity of the HLA-H*02:07 membrane-bound molecule to inhibit cytotoxic activity. HLA-G*01:01 K562-transduced cells were used as a positive control, and wild-type K562 cells were used as a reference (27).

The cytotoxicity assay was conducted using an xCELLigence Real-Time Cell Analyzer (Agilent Technologies). This assay allows monitoring of real-time target cell growth and lysis in a 96-well plate. Target cells are attached to well bottoms by a specific Ab. Each well bottom integrated microelectronic cell sensor arrays that measure impedance. Impedance at the well bottom varies according to target cell attachment. An increase in impedance reflects target cell growth, whereas a decrease reflects target cell lysis. Impedance is expressed as cell index (30).

Fifty thousand K562 target cells were seeded into each well coated with anti-CD71 Ab (IMT assay anti-CD71 tethering kit; Agilent Technologies). Target cell growth was monitored until the plateau phase was reached, and effector cells were then added (E:T ratio 2:1) (31).

Experiments were conducted with different effector cells: primary IL-2 activated PBMCs or the IL-2-independent NK92-MI cell line. Effector PBMCs were isolated from EDTA peripheral blood samples by density gradient centrifugation (Lymphoprep solution; STEMCELL Technologies) and cultured in stromal vascular fraction-supplemented RPMI. PBMCs were activated by 100 IU/ml of recombinant human IL-2 (rhIL-2) for 24 h (Life Technologies). The IL-2-independent NK92-MI cell line (CRL-2408; American Type Culture Collection) was cultured in NK MACS medium (Miltenyi Biotec) supplemented with serum from an AB donor.

Effector cells (25×10^3) were added (t0), and cell lysis was monitored every 15 min for 24 h (t24). Target cell lysis was assessed by the slope (cell index t0 to t24) and compared between assay and negative and positive controls. All experiments were conducted in a humidified incubator at 37°C with 5% CO₂. Each experiment was carried out in duplicate.

Characterization of the Denisovan HLA-H alleles

To investigate the Denisovan *HLA-H* content, all HLA class I-related reads were first isolated from the whole-genome sequences (32) using Bowtie 2 (33).

Initial *HLA-H* allelic assignment was performed with the PolyPheMe software (Xegen, Gemenos, France) (34) using the IPD-IMGT/HLA Database 3.43.0 as a reference (35) (Supplemental Table I). The *HLA-H* allelic typing accuracy of PolyPheMe software was previously validated (9, 36). Briefly, *HLA-H* typing was first validated on 25 individuals by comparing typing results from exome sequence data from the 1000 Genomes Project with typing results generated by a resequencing by next-generation sequencing on the original genomic DNA samples (9). Then, *HLA-H* typing accuracy was confirmed by comparing *HLA-H* full gene typing results by two independent next-generation sequencing methods and two independent types of software (PolyPheMe, Xegen; and AlloSeq Assign software, CareDx, Fremantle, Australia) (36).

Following this *HLA-H* allelic assignment, the two predicted *HLA-H* alleles were checked by remapping the HLA class I-related reads on the gene sequences for these two alleles. Reads with differences from the reference sequences were manually investigated to ensure specificity for the *HLA-H* locus and discarded when they were not specific (Supplemental Table I).

*HLA-H*02:07 worldwide distribution*

*HLA-H*02:07* worldwide distribution was visualized using  typing data from the 1000 Genomes Project (9, 37). Additionally, *HLA-H* and *HLA-G* types for 31 Melanesian individuals (38) defined with the PolyPheMe software (Xegen) were added to the data from the 1000 Genomes Project. *HLA-H*02:07* allele frequency was estimated for each subpopulation ($n = 26$) according to individual genotype using the GENE[RATE] program (39). *HLA-H*02:07* allelic frequency data were plotted on a world map using Mango software (<https://mangomap.com/>).

Statistical analyses

All association and correlation tests were performed with GraphPad Prism 9 software (GraphPad Software, La Jolla, CA). Differences between two modalities were tested using a Mann-Whitney *U* test. Kruskal-Wallis one-way ANOVA followed by a Dunn post hoc test was used to test more than two modalities.

Results

*HLA-H*02:07-transduced K562 cells express a membrane-bound HLA protein*

Lentiviral transduction of the K562 HLA-null cell line was used to investigate membrane-bound expression of the *HLA-H*02:07* allele.

Table I. Primer sequences used for lentiviral transduction expression assessment

Primer	Sequence
Albumin forward	5'-TTTCAGATGTCAGTGAAGAGA-3'
Albumin reverse	5'-TGGGGAGGCTATAGAAAATAAGG-3'
WPRE forward	5'-GTCCTTTCCATGGCTGCTC-3'
WPRE reverse	5'-CCGAAGGGACGTAGCAGA-3'
HLA-H forward	5'-CTGGGTGTTCTGTCCAGT-3'
HLA-H reverse	5'-AGGCGAGCCTAGATTCATCA-3'

Transduction with the *HLA-G*01:01* allele was used as a positive control.

K562 cell transduction efficiency was first validated by quantifying the lentivirus-specific transgene *WPRE* transcriptional activity (28). Both *HLA-H*02:07* K562 and *HLA-G*01:01* K562 cells were positive for *WPRE* transcription and showed quantitative PCR-positive amplification ($\Delta Ct^{WPRE} = -14$). Wild-type K562 cells displayed no signal for *WPRE* assays.

*HLA-H*02:07* K562 cell transduction was also validated by *HLA-H* transcriptional activity ($\Delta Ct^{HLA-H} = 10$). Wild-type K562 cells displayed no signal for *HLA-H* assays.

Expression of membrane-bound HLA molecules by transduced K562 cells was explored by flow cytometry using the anti-HLA class I W6/32 Ab and the anti- β_2 -microglobulin Ab. The *HLA-G*01:01* K562 cells used as a positive control for membrane-bound HLA expression displayed staining comparable to that of *HLA-H*02:07* K562 cells with W6/32-PE anti-HLA class I Ab (MFI-*HLA-H* K562 = 95; MFI-*HLA-G* = 70; MFI wild type = 19; Fig. 1) and B2M-01-PE anti- β_2 -microglobulin (data not shown).

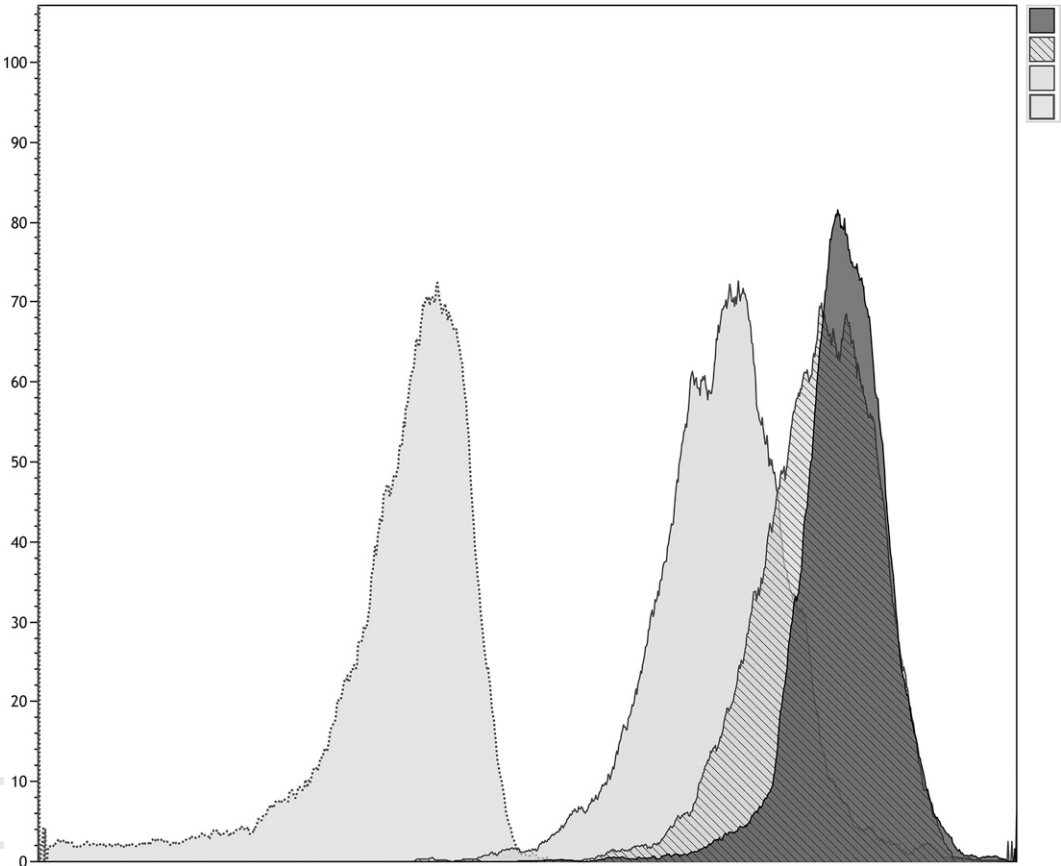
*HLA-H*02:07-transduced K562 cells diminish IL-2-activated PBMCs and IL-2-independent NK92 cell line cytotoxic activity*

Inhibition of cell lysis by immune effectors was analyzed using a real-time cytotoxicity assay conducted with K562 as target cells (positive control: *HLA-G*01:01* K562 cells; negative control: wild-type K562 cells; assay: *HLA-H*02:07* K562 cells) and IL-2-activated PBMCs or the IL-2-independent NK92-MI cell line as effector cells. Target lysis by IL-2-activated PBMCs showed no difference between *HLA-G*01:01* K562 cells and *HLA-H*02:07* K562 cells, whereas both were significantly different from wild-type K562 cells (Kruskal-Wallis test; $p = 0.002$; Fig. 2). *HLA-G* was shown to protect against NK cell cytotoxicity (27). Target lysis by the IL-2-independent NK92-MI cell line showed equivalent results (Kruskal-Wallis test; $p < 0.001$; Fig. 3). Primary data for the assay conducted with IL-2-activated PBMCs as effector cells are shown in Supplemental Fig. 1.

*HLA-H*02:07 is a Southeast Asian allele that is of Denisovan origin*

To define worldwide *HLA-H*02:07* distribution and investigate its potential evolutionary origin, we analyzed its frequency in populations from the 1000 Genomes Project as well as in Melanesian individuals (Supplemental Table II) because the 1000 Genomes Project does not cover Oceania. Worldwide *HLA-H*02:07* distribution (Supplemental Table III) is shown on a world map (Fig. 4). *HLA-H* in silico typing in 31 Melanesian individuals revealed an *H*02:07* allele frequency of 4.8% (Supplemental Table III). This worldwide analysis hence shows that the highest frequencies of *H*02:07* are observed in Southeast Asia, with a Chinese Dai population displaying the highest allelic frequency (37%).

FIGURE 1. Flow cytometry analysis with W6/32-PE anti-HLA class I Ab. *HLA-H*02:07* K562 cells (dark gray) and *HLA-G*01:01* K562 cells (striped gray) displayed similar staining as compared with wild-type K562 cells (light gray) staining. Unstained wild-type K562 cells are represented by a dashed line.



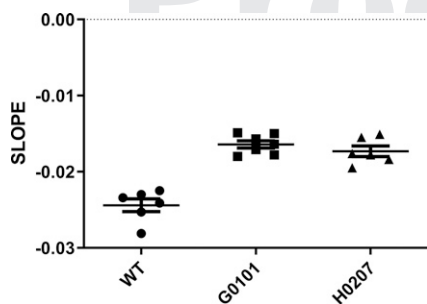


FIGURE 2. Dynamic monitoring of cell lysis (xCELLigence assay). Target cells were cultured into 96-well E-plates until the plateau phase was reached. IL-2-activated effector PBMCs were added (t0). Impedance at well bottoms was measured every 15 min for 24 h and normalized to baseline impedance values with medium only. Changes in impedance normalized to t0 are given as cell index, and cell viability was expressed by the slope from t0 to t24 and compared between assay (HLA-H*02:07 K562) and negative (wild-type K562) and positive controls (HLA-G*01:01 K562) (Kruskal-Wallis test; $p = 0.002$).

Because of this distribution and because *H*02:07* is in full LD with *HLA-A*11:01* [9] and Supplemental Table III) and *HLA-A*11:01* is of Denisovan origin (11), this raises the possibility that *H*02:07* is also of Denisovan origin. To test this possibility, *HLA-H* in silico typing was performed for the Denisovan genome with PolyPheMe software and showed the presence of *H*01:01-like* and *H*02:07-like* alleles. Careful remapping of the Denisovan HLA class I-related reads against reference gene sequences for *H*01:01* and *H*02:07* (Supplemental Table I; IPD-IMGT/HLA Database 3.43.0) confirmed the genotype *H*01:01/H*02:07* with 99.5% and 100% coverage, respectively. The Denisovan genome thus contains *H*02:07*. Together with the facts that this allele is in full LD with *HLA-A*11:01* in modern human populations (9) and that *HLA-A*11-containing* haplotypes are of Denisovan origin (11), this shows that *H*02:07* was brought into modern human populations by admixture with Denisovans.

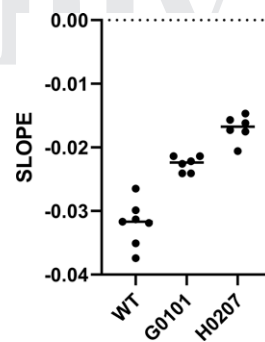


FIGURE 3. Dynamic monitoring of cell lysis (xCELLigence assay). Target cells were cultured into 96-well E-plates until the plateau phase was reached and NK-92 MI effector cells were added (t0). The method of determining the cell viability is the same as described in Fig. 2. Cell viability was compared between assay (HLA-H*02:07 K562) and negative (wild-type K562) and positive controls (HLA-G*01:01 K562) (Kruskal-Wallis test; $p < 0.001$).

Discussion

The pseudogene *HLA-H* locus displays 19 known alleles, two of which, *HLA-H*02:07* and *H*02:14*, potentially encode a full-length HLA protein (9). Because of *HLA-H*02:07* transcriptional activity and homology between its putative product and HLA-G molecule (9, 16), we hypothesized that the *H*02:07* allele encodes a functional, membrane-bound HLA molecule and contributes to immune regulation.

In this study, we show that *HLA-H*02:07* cDNA is expressed as a membrane-bound protein using an HLA-null erythroblast cell line (K562) transduced with *HLA-H*02:07* cDNA. Our results also support, using an in vitro cytotoxicity assay, that HLA-H*02:07 inhibits effector cells as efficiently as HLA-G. Thus, the HLA-H*02:07 protein may have an immunotolerant function, like the nonclassical molecules HLA-G, -E, and -F (20–26).



FIGURE 4. Worldwide *HLA-H*02:07* allele frequency (Mango software). Worldwide *HLA-H*02:07* frequency from the 1000 Genomes Project populations (9, 37) and from Melanesian individuals (38) (Frequencies are given in Supplemental Table II.)

We also explored *HLA-H*02:07* evolution and show that the allele was brought into modern humans through Denisovan admixture. Indeed, *HLA-H*02:07* is in full LD with *HLA-A*11*, an allele of Denisovan origin (11), and our in silico *HLA-H* typing of the Denisovan genome confirmed the presence of *H*02:07* in combination with the presence of *HLA-A*11*. Interestingly, although Denisovan admixture contributed to 4–6% of present-day Melanesian genomes (32) and a lot less in mainland Asia (<1% [40]), *HLA-H*02:07* is present in modern Asian genomes at a much higher frequency, with an allele frequency >35% in a Chinese population. Such a high *HLA-H*02:07* allelic frequency is consistent with adaptive introgression of either *H*02:07* or a variant at another locus that would be present on *H*02:07*-containing haplotypes. No selection test could be performed on *HLA-H* allelic sequences because coding polymorphism events are too uncommon (41). Similarly, the full LD between *HLA-H*02:07* and *HLA-A*11:01* (9) prevents precise selection analyses.

Although our results support that *HLA-H*02:07* is functional, it has no known receptor to date. The tolerogenic HLA-G molecule interacts with the inhibitory receptors LILRB1, LILB2, and KIR2DL4. Accordingly, the *HLA-H*02:07* molecule may be the ligand of different receptors with differential affinity. Interestingly, the fact that this variant was brought into modern humans through Denisovan admixture raises the possibility that one of the corresponding receptors may also have been brought by Denisovan admixture into modern populations. For example, killer Ig-like receptors are known receptors for HLA class I ligands, and one such variant, KIR3DS1*013, was a candidate for introgression from Denisovan (11).

The nonavailability of validated specific Ab directed against HLA-H is a main limitation of our study. Such a tool would allow HLA-H tissue expression exploration and functional confirmation by blocking experimental investigation.

Nevertheless, our study illustrates how HLA pseudogenes deserve dedicated studies to decipher their expression and their potential role in immune homeostasis.

Disclosures

Q:20 The authors declare that they have no competing interests.

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Key Points

- *HLA-H*02:07* encodes a membrane-bound protein.
- *HLA-H*02:07* inhibits human effector cell activity.
- *HLA-H*02:07* is of archaic origin.