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Research article

Roles of full-length and Δα1 HLA-G isoforms in NK-cell regulation and vascular SARS-CoV-2 infection☆

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ABSTRACT

Human Leukocyte Antigen-G (HLA-G) is a key immunomodulatory molecule with multiple isoforms, including recently identified variants lacking the α1 domain (HLA-G Δα1). The absence of the α1 domain may reduce immune inhibition, particularly of Natural Killer (NK) cells, which are often suppressed during viral infections such as SARS-CoV-2. This suggests a novel immune regulatory mechanism that could mitigate COVID-19-related complications, including coagulopathies. This study investigates the influence of full-length and Δα1 HLA-G isoforms in NK-cell activity in vitro during SARS-CoV-2 infection and whether their expression associates with viral persistence and coagulopathy in COVID-19 patients, using vascular specimens retrieved from surgical procedures.

RCC7 cell lines engineered to express either the full-length or Δα1 form of HLA-G were infected with Omicron SARS-CoV-2 to assess NK cell-mediated cytotoxicity in a co-culture system. Contrary to full-length, Δα1 HLA-G only partially inhibited NK activation, allowing greater cytotoxicity and higher IFN-I/II expression.

To evaluate clinical relevance, vascular tissues from coagulopathic COVID-19 patients were analyzed for in situ SARS-CoV-2 persistence and HLA-G isoform expression. SARS-CoV-2 negative tissues more frequently expressed Δα1 and displayed higher systemic interferon (IFNs) levels. Among infection-positive cases, Δα1 expression was also associated with stronger IFN-β and IFN-γ responses. These in vivo trends mirror in vitro findings, supporting a model in which full-length HLA-G dampens NK-mediated clearance of infected vascular cells, favoring viral persistence and potentially contributing to immunothrombosis, while Δα1 permits enhanced antiviral immunity.

Overall, these findings identify distinct immunomodulatory roles for HLA-G isoforms in SARS-CoV-2 infection. The Δα1 variant exhibits reduced inhibitory potency, partially preserving NK-cell antiviral functions and modulating interferon responses, whereas full-length HLA-G may facilitate vascular viral persistence and coagulopathy. HLA-G isoform profiling may thus represent a novel biomarker and therapeutic target in COVID-19 associated vascular pathology.

Abbreviations: HLA, Human Leukocyte Antigen; NK, Natural Killer; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19, Coronavirus Disease 2019; ccRCC, clear cell Renal Cell Carcinoma; LDH, lactate dehydrogenase; IFN, interferon; ACE2, Angiotensin Converting Enzyme 2; CD147, Cluster of Differentiation 147; TMPRSS2, Transmembrane Protease Serine 2; PLpro, papain-like protease; RT-q-PCR, Reverse Transcription quantitative Polymerase Chain Reaction; WT, Wild Type; BLS-3, biosafety level-3; MOI, multiplicity of infections; cDNA, complementary DNA; SCV2pos/neg, SARS-CoV-2 positivity/negativity.

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1. Introduction

Human leukocyte antigen-G (HLA-G), a major histocompatibility complex class Ib antigen with distinctive features compared to classical HLA class Ia molecules, exhibits low protein variability, restricted tissue expression, immunosuppressive activity and exists in seven different isoforms generated by alternative splicing of the primary transcript. These include four membrane-bound forms (HLA-G1, −G2, −G3, −G4) and three soluble forms (HLA-G5, −G6, −G7) [1]. These isoforms differ in their extracellular domain composition (α1, α2, α3). The α1–α2 platform forms a peptide-binding groove typical of MHC class I molecules; however, in HLA-G peptide binding is thought to primarily support correct folding and stability of the complex. The immunosuppressive/tolerogenic effects of HLA-G are mainly mediated by engagement of inhibitory receptors on immune cells rather than by classical antigen presentation.

Beyond the seven canonical isoforms, additional spliced variants have been identified in tumors, reflecting HLA-G transcriptomic heterogeneity and its impact on immune checkpoint regulation. The HLA-G Δα1 isoform (257/526-) is the only isoform described that lacks the α1 extracellular domain, first identified in cell renal cell carcinoma (ccRCC) and subsequently in trophoblasts [2,3]. Nevertheless, HLA-G expression might have been underestimated, since currently available antibodies predominantly target the α1 domain, making Δα1 undetectable by conventional assays. Functionally, the absence of the α1 domain disrupts the canonical peptide-binding platform and likely compromises formation of a classical MHC-I–like complex, including efficient association with β2-microglobulin, thereby limiting canonical antigen presentation to T cells. Instead, Δα1 displays immune-stimulatory properties toward Natural killer (NK) and T cells, in contrast to classical HLA-G isoforms that are largely inhibitory [2]. The preservation of NK cytotoxicity thus represents a distinctive relevant feature of HLA-G Δα1. NK cells play a pivotal role in the early control of viral infections by rapidly detecting and eliminating infected cells through a combination of cytotoxic activity and cytokine production. NK cells are rapidly recruited to the lung during SARS-CoV-2 infection, where they contribute to early viral control through cytotoxicity and interferon (IFN)-γ production, but accumulating evidence indicates that their number and function are profoundly dysregulated in severe coronavirus disease 2019 (COVID-19). In vitro, Bortolotti et al. demonstrated that expression of SARS-CoV-2 Spike 1 in lung epithelial cells upregulates HLA-E and engages the inhibitory receptor NKG2A on NK cells, resulting in reduced degranulation and IFN-γ secretion and an exhausted NK-cell phenotype that may facilitate viral persistence and immunopathogenesis [4]. In patients, SARS-CoV-2 infection is associated with marked alterations in NK-cell frequency and phenotype within the broader innate immune response, and proposed that a prompt and quantitatively adequate NK-cell response is critical for effective viral clearance and for limiting the progression toward hyperinflammation [5]. NK cells are central early effectors against SARS-CoV-2 but are actively subverted by viral immune-evasion mechanisms and the inflammatory milieu, so that failure to maintain NK-cell effector function may predispose to higher viral load and severe COVID-19. SARS-CoV-2–induced dysfunction of NK cells is increasingly recognized as a potential contributor to the coagulopathies observed in COVID-19 [6], through impaired immune surveillance of infected and activated vascular and immune cells, and through dysregulated crosstalk with platelets and the endothelium [7]. In COVID-19, NK cells are numerically reduced and often display an exhausted or inhibitory receptor–skewed phenotype, with decreased cytotoxicity and IFN-γ production [8]; this may limit their ability to eliminate virally infected endothelial cells and monocytes that drive tissue factor expression, and thrombin generation. Functionally impaired NK cells may also fail to clear activated platelets and microthrombi, favoring persistence of a procoagulant milieu rich in IL-6, TNF, and other cytokines that further suppress NK-cell effector functions, creating a vicious circle between innate immune paralysis and hypercoagulability [9]. Thus, SARS-CoV-2–mediated NK-cell exhaustion and quantitative defects are not only important for defective viral control, but may also indirectly promote endothelial activation, immunothrombosis, and the characteristic micro- and macrovascular thrombotic complications of COVID-19 [8].

These complications are frequently associated with an increased incidence of out-of-hospital sudden death [10]. Importantly, SARS-CoV-2 displays broad tropism beyond the respiratory tract, being detected in several organs, due to the wide distribution across extrapulmonary tissues of its entry receptors, Angiotensin Converting Enzyme (ACE) 2 and cluster of differentiation 147 (CD147) [11–13], together with integrins and TMPRSS2 (Transmembrane Protease, Serine 2) as some of the main viral co-receptors [14,15].

SARS-CoV-2 presence in the digestive tract of asymptomatic patients with hypercoagulable states and bowel ischemia has been documented [12], supporting the concept of extrapulmonary involvement/persistence that may contribute to thrombotic complications through tissue and endothelial dysfunction and dysregulation of anticoagulant pathways, rather than implying a direct causal effect of viral spread per se.

Taking into consideration the peculiar role of HLA-G Δα1, this isoform might provide valuable insights into compensatory immune mechanisms during COVID-19 disease.

Among the molecular contributors in coagulation decompensation in COVID-19, our group identified the SARS-CoV-2 papain-like protease (PLpro) enzyme as a driver of coagulation imbalance, in association with increased CD147 expression [16]. This observation is particularly relevant since HLA-G expression can be modulated by CD147 [13].

Investigating both classical and non-classical HLA-G isoforms is therefore essential for understanding the complex interplays between viral infections and the host immunity. The diverse immunomodulatory functions of HLA-G isoforms strongly suggest that their differential expression contributes to the immune dysregulation observed in viral diseases. In this context, novel isoforms, such as Δα1, may provide valuable insights into compensatory immune mechanisms that could be harnessed therapeutically. Comprehensive profiling of HLA-G isoforms could thus clarify how viruses evade immune surveillance, facilitate persistence and disease progression, revealing new opportunities for targeted intervention.

The objective of this work was to address the roles of full-length and Δα1 HLA-G isoforms in NK-cell regulation and vascular SARS-CoV-2 infection. We focus on how the structural difference introduced by the loss of the α1 domain may translate into divergent immunomodulatory effects, with potential implications for endothelial involvement and COVID-19–associated thrombo-inflammatory complications.

2. Materials and methods

2.1. Cell lines

The RCC7 Tumor cell line derived from primary RCC, obtained from a clear cell renal cell carcinoma patient, collected with the prior consent of the donor and anonymized, was kindly gifted by Prof. Edgardo D. Carosella, Hôpital Saint-Louis, Paris [17]. The RCC7 line does not express HLA-G (WT), thus it was previously transduced with lentivirus containing HLA-G1 or HLA-G Δα1 cDNA, generating a stable cell line expressing high levels of either HLA-G1 or Δα1 isoform (named CB1 and CB6, respectively), as previously described [17]. WT and transduced RCC7 cells were grown in DMEM (Gibco™ Thermo Fisher Scientific™, Waltham, MA, U.S.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Merck), 1% amphotericin B 250 μg/ml (Gibco™) and 1% gentamicin 10 mg/l (Gibco™).

Human NK-92 natural killer cell line (ATCC® CRL-2407TM) was used, expanded in Alpha Minimum Essential Medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM 2-mercaptoethanol, and 20% fetal bovine serum.

Cultures were maintained at 37 °C and 5% CO_2 and routinely tested

for mycoplasma contamination.

2.2. Virus infection and propagation

Omicron SARS-CoV-2, kindly provided by Prof. A. Caruso, University of Brescia, Italy [18], was propagated and titrated by quantitative-Polymerase Chain Reaction (q-PCR) assay on VeroE6 cells (ATCC, Manassas, VA, Number CRL-1586). The virus was propagated in Vero E6 cells and titrated using the plaque assay as previously described [18]. RCC7 cells were infected with a multiplicity of infection (MOI) of 0.1 for 2 h at 37 °C, following established protocols [18,19]. Supernatants from infected cells were collected 48 h post-infection and used for infection experiments. All procedures were conducted in a biosafety level-3 (BSL-3) laboratory.

2.3. Plaque assay for viral titration

To determine the levels of infective SARS-CoV-2 released by infected cells, a plaque assay was performed. Supernatants from infected RCC7 cells were used to infect Vero E6 cells. Five days post-infection, the cells were fixed with methanol, stained with 0.1% crystal violet, and the plaques were counted and expressed as PFU/ml [18]. All experiments were conducted in triplicate.

2.4. NK cytotoxicity assay (Lactate dehydrogenase assay)

NK cell-mediated cytotoxicity was detected by lactate dehydrogenase (LDH) assay, using the Lactate Dehydrogenase Activity Assay kit (MAK066-1 K1-Sigma-Aldrich) according to the manufacturer's instructions, as previously reported [20].

The extent of lactate dehydrogenase (LDH) release was used to measure the degree of NK cell activity after the co-culturing with target RCC7, either WT or transduced with both the isoforms of HLA-G (CB1 and CB6).

LDH release was detected measuring absorbance at 490 nm. The lysis of target cells that were not co-cultured with NKs served as Target Cell Maximum LDH release control (positive control).

2.5. Patients

18 Patients affected by coagulopathies (peripheral arterial thrombosis) were enrolled at the Internal Medicine Unit of the Sant'Anna University hospital in Ferrara. The entire cohort presented with arterial thrombosis and required surgical intervention for thrombus removal. During surgical intervention, the endothelial tissues were collected from each subject and used for total RNA extraction, as previously described [16]. All patient sample collections were conducted in accordance with the Declaration of Helsinki and approved by the institutional ethics committee (Number: 540/2020/Oss/AOUFe—20 may 2020), with written informed consent obtained from all participants prior to enrollment. 7 patients tested positive for SARS-CoV-2 oropharyngeal swab within 6 months of the coagulopathy event and 11 patients had never reported a SARS-CoV-2 positive oropharyngeal swab. No subject was vaccinated for SARS-CoV-2

2.6. RT-PCR and RT-q-PCR

Total RNA extraction in Trizol™ Reagent (LifeTechnologies), followed by reverse transcription (RT) performed using SuperScript IV VILO (Thermo Fisher, Milan, Italy) was performed on the collected samples, following manufacturer instructions.

The presence of HLA-G α1 domain was detected using PCR (Polymerase Chain Reaction), following the amplification protocol described by Tronik-Le Roux et al. [21]. The primers employed for amplification were G257F (5′-GGA AGA GGA GAC ACG GAA CA) and G526R (5′-CCT TTG TTC AGC CAC ATT GG) to detect the presence of α1, and the combination of G526F (5′-CCA ATG TGG CTG AAC AAA GG) and TAGR (5′-TTA CTT GTC ATC GTC GTC CTT GTA GTC) to specifically identify Δα1 isoform.

Quantification of SARS-CoV-2 genomes through RT-q-PCR was done by amplifying the S gene with a PowerUp SYBR Green Master Mix (Thermo Fisher, Milan, Italy) using the primers RBD-qF1: 5′-AATGGTTTAACAGGCACAGG-3′ and RBD-qR1: 5′-CTCAAGTGTCTGTG-GATCACG-3′, as previously described [16]. For absolute quantification, a dsDNA fragment (gBlock, Integrated DNA Technologies, Leuven, Belgium), Coralville, IA, USA) based on the RBD sequence was used to create a standard curve ranging from 10^8 to 10^2 copies.

Expression levels of type I (IFN-α and IFN-β) and type II (IFN-γ) interferons in NK cells after the co-culture with the HLA-G isoforms and viral infection, were quantified by RT-qPCR using human-specific assays. The following PrimeTime assays (Integrated DNA Technologies, Leuven, Belgium) were employed: IFN-α (Hs.PT.58.46311748.g), IFN-β (Hs.PT.58.39481063.g), and IFN-γ (Hs.PT.58.3781960.g).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Plasma levels of type I and type II interferons from coagulopathic COVID-19 patients were quantified in triplicate using a standard enzyme-linked immunosorbent assay (ELISA). Measurements were performed with commercially available kits from MyBioSource, following the manufacturer's instructions. The specific assay kits used were: IFN-α (MBS2019548), IFN-β (MBS2513798), and IFN-γ (MBS161542). This approach allowed for sensitive and reproducible detection of circulating interferon levels across patient samples.

2.8. Statistical analysis

Data were analyzed using GraphPad Prism 10 (GraphPad Software, California, USA). Mean values from in vitro infection experiments and in vivo clinical data from coagulopathic patients were compared using Student's *t*-test and using Kruskal-Wallis followed by Dunn correction, for multiple comparisons. The distribution of coagulopathic patients expressing HLA-G isoforms according to SARS-CoV-2 positivity or negativity was evaluated using Fisher's exact test. Respective statistical analysis is provided in the corresponding figure legends. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. HLA-G Δα1 preserved NKs cytotoxicity, reducing viral spread

Firstly, WT and transduced RCC7 cell lines (CB1 and C6, expressing full-length and the Δα1 isoform, respectively) were tested for their susceptivity to Omicron SARS-CoV-2 infection (Fig. 1).

The viral titration by plaque assay did not show any differences in Omicron SARS-CoV-2 infection susceptibility among the three cell lines tested, supporting no direct role of HLA-G isoforms in SARS-CoV-2 infection.

To assess the effect of HLA-G isoforms on NK cells activation during SARS-CoV-2 infection, we co-cultured NK cells with SARS-CoV-2–infected RCC7 WT, CB1 and CB6 cells, and evaluated NK cell cytotoxicity (Fig. 2).

Notably, whereas RCC7 WT exhibited a marked enhancement of NK cell cytotoxic activity following SARS-CoV-2 infection compared to mock (Fig. 2a; 75% vs 30%, respectively; $p < 0.001$, Student's *t*-test), only a slight activation of NK cell killing was observed in RCC7 CB1 (full-length HLA-G) under the same conditions (Fig. 2a; 35% infected vs 28% mock, $p < 0.05$, Student's *t*-test), indicating a possible impairment of NKs efficacy associated with full-length HLA-G expression. Interestingly, the presence of HLA-G Δα1 isoform (RCC7 CB6) seems to partially preserve NK cell functionality against SARS-CoV-2 (Fig. 2a; 58% infected vs 33% mock, $p < 0.01$, Student's *t*-test).

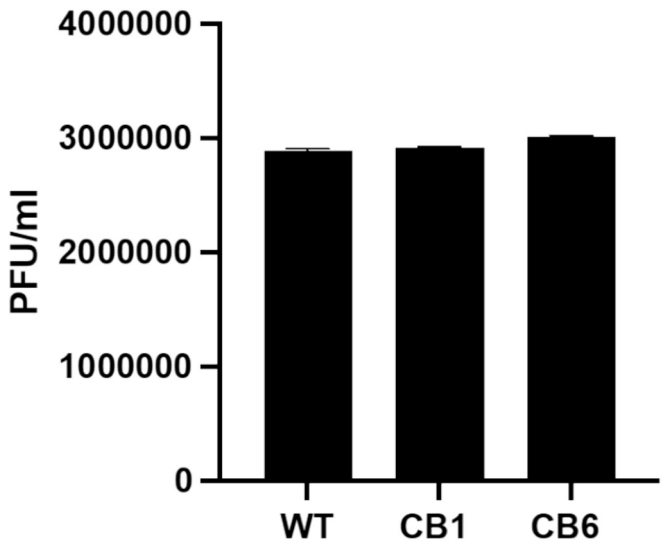

Fig. 1. Omicron SARS-CoV-2 titration in infected RCC7 cell line expressing no HLA-G (WT), full length HLA-G (CB1) and HLA-G Δα1 (CB6). Viral titration is reported as mean ± SD of three independent experiments.

As a proof-of concept, the infection rate was assessed in the co-culture system by titration. As shown in Fig. 2b, cells expressing HLA-G Δα1 isoform (RCC7 CB6) or with no HLA-G expression (RCC7 WT) were characterized by a similar infection rate, compared to the infected cell expressing full-length HLA-G molecule, where a higher SARS-CoV-2 infection rate was observed. (Fig. 2b; 5.14 x 10^5 PFU/ml CB6 vs 2.43 x 10^6 PFU/ml CB1 vs 1.89 x 10^5 PFU/ml WT, $p < 0.05$, Kruscal-Wallis test).

These findings support the role of HLA-G Δα1 in limiting viral spread through immune modulation mediated by effector cells such as NK cells.

To assess the effects of the full-length and Δα1 HLA-G isoforms on NK-cell activation, we measured interferon secretion levels in the co-culture system. since type I and II interferons are key regulators of NK-cell maturation, activation, and cytotoxic licensing, interferon expression serves as a sensitive readout of how different HLA-G isoforms modulate NK-cell effector functions. The Δα1 HLA-G variant lacks the α1 extracellular domain, which may reduce interactions relying on α1-mediated recognition—such as KIR2DL4 binding—thereby potentially altering NK-cell functional outputs [22]; conversely, engagement of ILT2/ILT4 (LILRB1/2), which primarily involves the α3 domain (and β2-microglobulin for LILRB1) [23,24], may be less directly affected. By quantifying interferons, particularly IFN-γ—a key marker of NK-cell activation—we can determine whether each HLA-G isoform suppresses or permits NK-cell functional activation, revealing isoform-specific immunomodulatory effects that may not be detected by cytotoxicity assays alone.

In contrast to IFN-α (Fig. 3a), we observed higher IFN-β and IFN-γ mRNA expression (Fig. 3b, 3c) in culture supernatants of NK cells co-cultured with SARS-CoV-2–infected cells either expressing the HLA-G Δα1 isoform (RCC7 CB6) or lacking HLA-G expression (RCC7 WT), compared with infected cells expressing the full-length HLA-G molecule (RCC7 CB1) (Fig. 3b: 20-fold CB6 vs 1-fold CB1, $p < 0.05$, Kruskal-Wallis test; Fig. 3c: 15-fold CB6 and 12-fold WT vs 2-fold CB1, $p < 0.05$, Kruskal-Wallis test). These findings support distinct mechanistic effects of the full-length and Δα1 HLA-G isoforms, which translate into different NK-cell activation profiles.

3.2. *SARS-CoV-2 in situ infection is associated with HLA-G Δα1 in coagulopathic patients*

Based on our in vitro findings, HLA-G–dependent modulation of NK-cell activation emerges as a plausible link between SARS-CoV-2 infection and the development of coagulopathies. We observed that SARS-CoV-2–infected RCC7 cells expressing the Δα1 HLA-G isoform (RCC7 CB6), or lacking HLA-G altogether (RCC7 WT), induced higher levels of type I and type II interferons in NK-cell culture supernatants compared to infected cells expressing the full-length HLA-G molecule, consistent with a more robust NK-cell activation profile in the absence of intact HLA-G–mediated inhibitory signaling. Since dysregulated NK-cell responses and interferon signaling have been implicated both in antiviral control and in immunothrombosis, these data suggest that the relative predominance of full-length versus Δα1 HLA-G isoforms on target cells could shape the balance between effective viral clearance and pathological inflammation. We therefore decided to investigate the expression pattern of HLA-G isoforms in coagulopathic patients after SARS-CoV-2 infection, to test the hypothesis that an altered HLA-G isoform repertoire, favoring immunosuppressive full-length HLA-G or failing to upregulate Δα1 variant, might contribute to impaired NK-cell activation, inadequate interferon responses, and a prothrombotic innate immune milieu in vivo.

The patients were subdivided according with SARS-CoV-2 infection in vascular specimens, analyzed by RT-q-PCR for RBD domain [16] and on HLA-G isoform expression. We detected in situ infection in 7 of the 18 patients examined. We observed a higher frequency of in situ infection in patients expressing at least one positivity for HLA-G full length mRNA (257/526 +/+; 257/526 +/-), while HLA-G Δα1 mRNA (257/526 −/-)

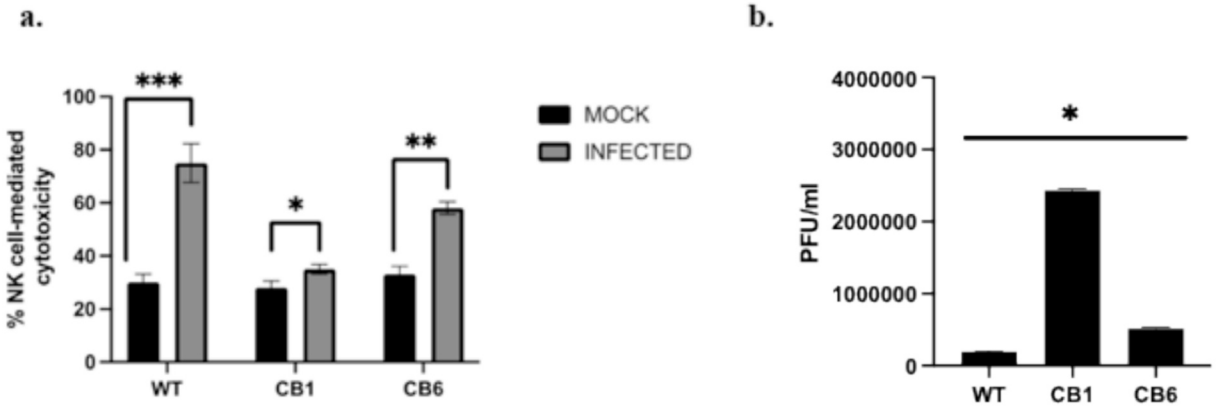

Fig. 2. Effect of RCC7 cells expressing no HLA-G (WT), full-length HLA-G (CB1), or HLA-G Δα1 (CB6) on a) NK cell activity and b) infection rate. **(a)** NK cell cytotoxic activity in uninfected (MOCK) and SARS-CoV-2–infected cells expressing the different HLA-G mRNA isoforms. **(b)** SARS-CoV-2 titration by plaque assay assessing the impact of these specific HLA-G isoforms on viral replication after the co-culture. Student's *t*-test and Kruskal-Wallis followed by Dunn's correction were used for statistical analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

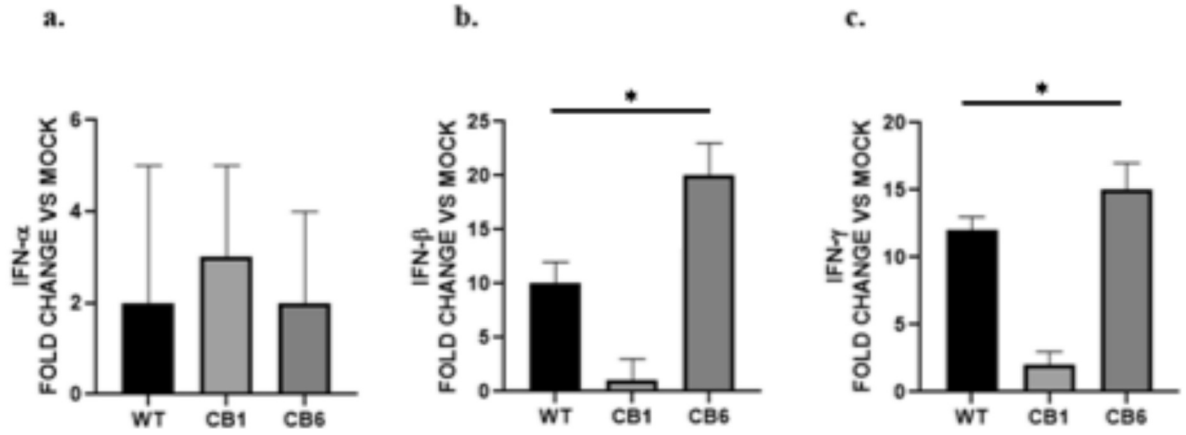

Fig. 3. Effect of RCC7 cells expressing no HLA-G (WT), full-length HLA-G (CB1), or HLA-G Δα1 (CB6) on NKs activation during infection, by assessing a) IFN-α, b) IFN-β and c) IFN-γ mRNA levels. **(a)** IFN-α, **(b)** IFN-β, and **(c)** IFN-γ expression in SARS-CoV-2–infected NK cells, shown as fold change relative to mock controls, after co-culture with the different HLA-G isoforms. Kruskal-Wallis followed by Dunn's correction was used for statistical analysis. * $p < 0.05$.

was more represented in specimens without in situ SARS-CoV-2 infection, even without reaching significance (Fig. 4; 10% vs 20%, respectively, $p = 0.074$, Fisher's Exact test).

As proof of concept, we evaluated the levels of type I and II interferons in the plasma samples of the 18 patients. We observed that SARS-CoV-2 in situ negative patients showed a slightly higher level of IFN-β and significantly increased level of IFN-γ (Fig. 5; SCV2neg vs SCV2pos for IFN-γ, $p < 0.05$, Student's *t*-test) in comparison with SARS-CoV-2 in situ positive patients.

When we subdivided the SARS-CoV-2 in situ positive patients according to HLA-G isoform mRNA expression (Fig. 6), we observed that the patients with HLA-G Δα1 mRNA (257/526 −/-) expression presented higher levels of IFN-γ in comparison with patients expressing at least one positivity for HLA-G full length mRNA (257/526 +/+; 257/526 +/-) (Fig. 6; IFN-γ, $p < 0.05$, Kruskal-Wallis test).

4. Discussion

This study underscores the critical and multifaceted role that HLA-G isoforms play in modulating immune responses during SARS-CoV-2 infection, with particular emphasis on the distinct functions of the full-length HLA-G and the alpha1-deleted isoform, HLA-G Δα1. Understanding the role of HLA-G isoforms is essential for unraveling the complex interactions between viral infections and host immunity. Given its diverse immunomodulatory functions, the expression and distribution of HLA-G are likely pivotal in the immune dysregulation commonly observed in viral diseases. Specifically, characterizing less studied isoforms, such as Δα1, may reveal compensatory mechanisms that help preserve immune function during infection.

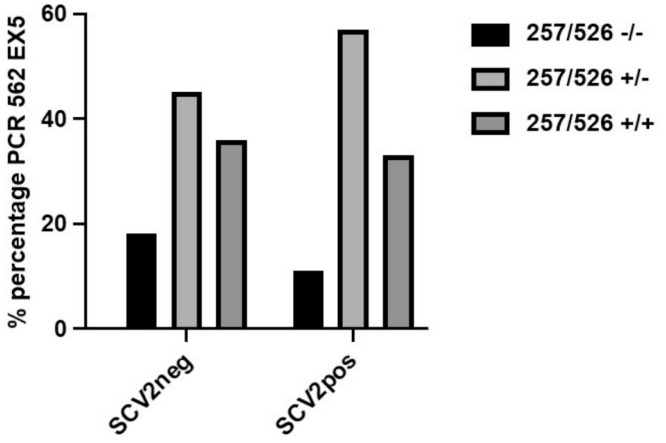

Fig. 4. Percentage of patients expressing the two HLA-G isoforms in vascular specimens stratified by in situ SARS-CoV-2 positivity (SCV2pos) or negativity (SCV2neg). Expression of the HLA-G Δα1 isoform is defined as 257/526 −/-, whereas patients exhibiting at least one allele positive for the full-length HLA-G transcript are classified as 257/526 +/+ or +/-.

In this study, we provide evidence that HLA-G isoforms differentially modulate NK-cell responses to SARS-CoV-2 and may influence viral persistence and coagulopathy in infected patients. Our in vitro experiments first demonstrated that neither full-length nor Δα1 HLA-G expression alters intrinsic cellular susceptibility to Omicron SARS-CoV-2 infection, as viral titers were comparable across WT, CB1, and CB6 RCC7 cell lines. This finding aligns with previous studies showing that SARS-CoV-2 entry and replication are primarily governed by ACE2/TMPRSS2 expression and viral–host membrane fusion mechanisms rather than by immune checkpoint molecules such as HLA-G [25,26].

Despite the lack of impact on infectivity, HLA-G isoforms markedly shaped NK-cell effector responses during SARS-CoV-2 infection. Consistent with the well-established role of HLA-G in immune tolerance [2], SARS-CoV-2–infected RCC7 cells expressing full-length HLA-G strongly suppressed NK-cell cytotoxicity, whereas NK activation was only partially attenuated by the Δα1 isoform. The preservation of NK cytotoxicity and the increased interferon secretion observed in CB6 (Δα1) and WT cultures are consistent with an overall reduction of inhibitory signaling. This effect may involve α1-dependent receptor engagement (e.g., KIR2DL4), whereas ILT2/ILT4 (LILRB1/2) interactions—primarily mediated by the α3 domain (and β2-microglobulin for LILRB1)—may be less directly affected; any impact on LILRB signaling would therefore be indirect and cannot be inferred from these functional assays alone. Future studies using receptor-blocking antibodies and/or receptor-specific binding assays will be required to dissect the contribution of individual inhibitory receptors. These findings are consistent with the broader concept the concept reported by Bortolotti et al. that SARS-CoV-2 can restrain NK activation through inhibitory checkpoint pathways (via HLA-E/NKG2A) and suggest that distinct HLA-G isoforms may differ in their inhibitory potency. The increased IFN-I and IFN-II responses in CB6 and WT co-cultures further support a model in which Δα1-expressing cells allow more effective NK-mediated antiviral responses, potentially limiting viral spread, as reflected by the lower post–co-culture viral titers relative to full-length HLA-G cells.

Based on these mechanistic insights, we investigated whether HLA-G isoform expression correlates with SARS-CoV-2 persistence and coagulation abnormalities in vivo. In vascular specimens from 18 patients, in situ SARS-CoV-2 infection was detected in 7. Notably, full-length HLA-G mRNA was more frequently expressed among SARS-CoV-2–positive

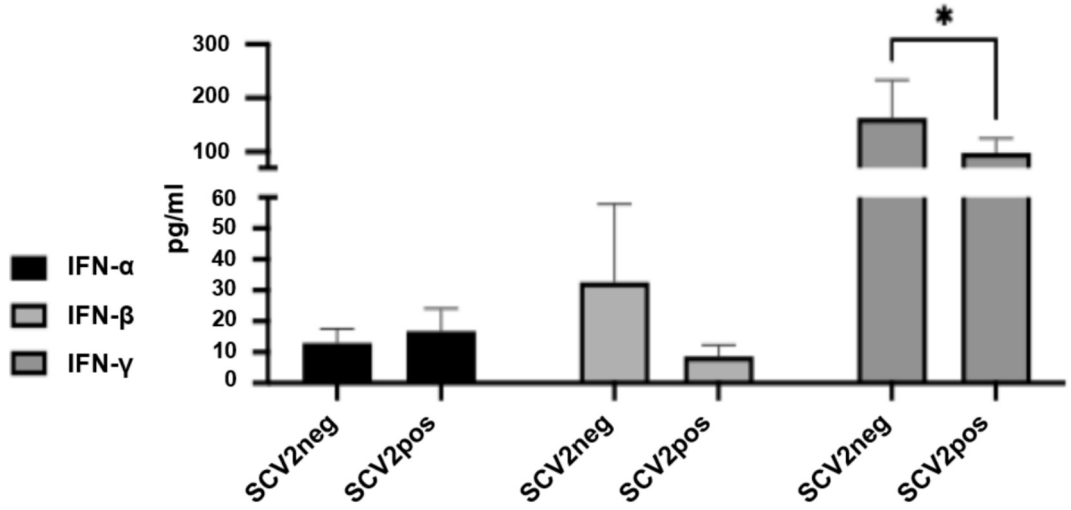

Fig. 5. Assessment of IFN-α, IFN-β, and IFN-γ plasma levels in patient cohorts negative or positive for SARS-CoV-2 in situ detection (SCV2neg and SCV2pos). Student's *t*-test was used for statistical analysis. * $p < 0.05$.

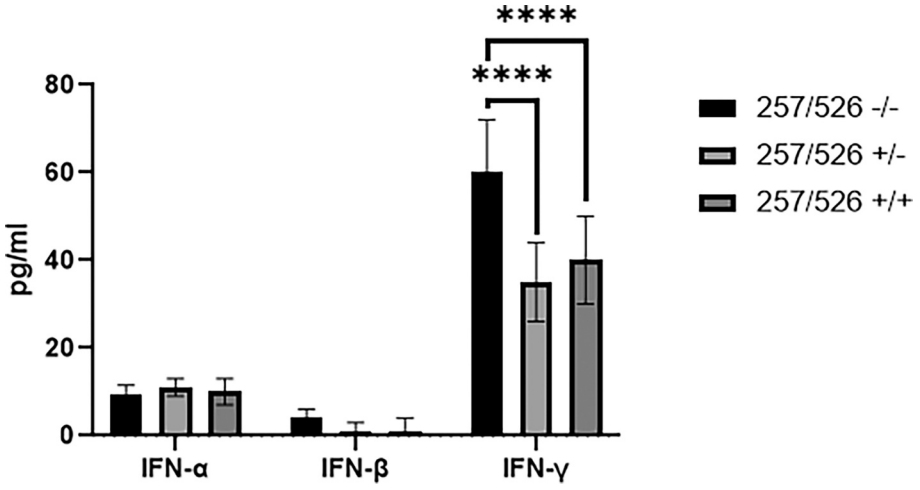

Fig. 6. IFN-α, IFN-β, and IFN-γ protein levels in patients positive for in situ SARS-CoV-2 detection (SCV2pos), grouped according to HLA-G isoform mRNA expression. HLA-G Δα1 expression is defined as (257/526 −/-), whereas patients exhibiting at least one allele positive for the full-length HLA-G mRNA (257/526) are classified as +/+ or +/-. Kruskal-Wallis followed by Dunn's correction was used for statistical analysis. * $p < 0.05$.

tissues, whereas Δα1 HLA-G transcripts predominated in infection-negative samples. Although not statistically significant, this trend mirrors our in vitro findings, suggesting that expression of full-length HLA-G may dampen NK-mediated clearance of infected endothelial or perivascular cells, potentially enabling viral persistence within the vascular compartment. This hypothesis is consistent with reports showing that impaired NK responses correlate with severe COVID-19 and thrombotic complications [27,28], and aligns with studies implicating endothelial SARS-CoV-2 infection as a contributor to immunothrombosis [29].

The systemic interferon profile of patients provided further support for a functional divergence between HLA-G isoforms. SARS-CoV-2 in situ–negative individuals displayed slightly higher IFN-β and significantly increased IFN-γ levels compared with infection-positive patients, in agreement with the concept that intact NK activity and interferon responses contribute to viral clearance. Moreover, among SARS-CoV-2 in situ–positive patients, those expressing the Δα1 HLA-G isoform exhibited higher IFN-β and IFN-γ levels than individuals expressing full-length HLA-G, highlighting a possible role for HLA-G isoform balance in shaping antiviral immunity within vascular tissues. These observations aligns with our in vitro data and reinforce emerging evidence that dysregulated interferon signaling plays a dual role in COVID-19 pathogenesis, promoting early viral control yet potentially contributing to endothelial inflammation and thrombosis when inadequately regulated [30,31].

Taken together, our findings reveal a previously underappreciated interplay between HLA-G isoforms, NK-cell activation, and SARS-CoV-2–associated vascular pathology. Full-length HLA-G appears to inhibit NK-cell cytotoxic activity, potentially reducing antiviral surveillance within vascular tissues and contributing to an immunological milieu permissive to vascular dysfunction and coagulopathy. In contrast, the Δα1 isoform partially preserves NK effector functions and supports more robust interferon production, which may enhance viral clearance but also modulate local inflammatory and thrombotic processes. While our patient cohort is limited in size and further studies are needed to clarify causality and mechanistic pathways, these results open new avenues for understanding immune dysregulation in COVID-19 and suggest that HLA-G isoform profiling could serve as an informative biomarker of vascular infection susceptibility and thrombotic risk.

6. Institutional review board statement

The study was conducted in accordance with the Declaration of

Helsinki and approved by the Ethics Committee of Sant'Anna University Hospital in Ferrara (protocol code 540/2020/Oss/AOUFe—20 May 2020).

7. Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

G. Schiuma: Writing – original draft, Methodology, Investigation, Data curation. **D.Tronik-Le Roux:** Methodology, Investigation, Formal analysis, Data curation. **S. Beltrami:** Methodology, Investigation, Formal analysis. **M. Ferraresi:** Methodology, Investigation, Formal analysis. **G. Pezzi:** Methodology, Investigation, Formal analysis. **G. Strazzabosco:** Methodology, Investigation, Formal analysis. **V. Gentili:** Methodology, Investigation, Formal analysis. **A. Passaro:** Writing – original draft, Data curation. **J.M.Sanz Molina:** Writing – original draft, Data curation. **M. Baroni:** Writing – original draft, Methodology, Funding acquisition, Formal analysis. **D. Bortolotti:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **E.D. Carosella:** Writing – review & editing, Conceptualization. **R. Rizzo:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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