



HLA-C-restricted viral epitopes are associated with an escape mechanism from KIR2DL2⁺ NK cells in Lassa virus infection

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ARTICLE INFO

Article history:

Received 8 November 2018

Received in revised form 22 January 2019

Accepted 22 January 2019

Available online 30 January 2019

Keywords:

Lassa virus

NK cells

KIR-L

HLA-C

Viral escape

ABSTRACT

Background: Lassa virus (LASV) is the etiologic agent of an acute hemorrhagic fever endemic in West Africa. Natural killer (NK) cells control viral infections in part through the interaction between killer cell immunoglobulin-like receptors (KIRs) and their ligands. LASV infection is associated with defective immune responses, including inhibition of NK cell activity in the presence of MHC-class 1⁺-infected target cells.

Methods: We compared individual KIR and HLA-class 1 genotypes of 68 healthy volunteers to 51 patients infected with LASV in Sierra Leone, including 37 survivors and 14 fatalities. Next, potential HLA-C1, HLA-C2, and HLA-Bw4 binding epitopes were in silico screened among LASV nucleoprotein (NP) and envelope glycoprotein (GP). Selected 10-mer peptides were then tested in peptide-HLA stabilization, KIR binding and polyfunction assays.

Findings: LASV-infected patients were similar to healthy controls, except for the inhibitory *KIR2DL2* gene. We found a specific increase in the *HLA-C1:KIR2DL2* interaction in fatalities (10/11) as compared to survivors (12/19) and controls (19/29). We also identified that strong of NP and GP viral epitopes was only observed with HLA-C molecules, and associated with strong inhibition of degranulation in the presence of KIR2DL⁺ NK cells. This inhibitory effect significantly increased in the presence of the vGP₄₂₀ variant, detected in 28.1% of LASV sequences.

Interpretation: Our finding suggests that presentation of specific LASV epitopes by HLA-C alleles to the inhibitory KIR2DL2 receptor on NK cells could potentially prevent the killing of infected cells and provides insights into the mechanisms by which LASV can escape NK-cell-mediated immune pressure.

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1. Introduction

Lassa virus (LASV) of the *Arenaviridae* family is the etiologic agent of the viral hemorrhagic disease Lassa fever (LF). The primary natural reservoir for LASV is the African multimammate mouse (*Mastomys natalensis*), and transmission to humans occurs via direct contact with rat excreta or by contact with or ingestion of contaminated materials [1,2]. Human-to-human transmission occurs via exposure to blood or bodily fluids, and nosocomial infections are of concern, particularly in low-resource settings [3]. Due to the wide geographical distribution as well as the high reproduction rate and commensal nature of *Mastomys* in Africa, LF appears to be endemic to Nigeria, Liberia, Sierra Leone, Guinea, and Mali, and likely exists in focal pockets across West Africa

[4]. In some areas of Liberia and Sierra Leone, 10–15% of all hospital admissions are due to LF indicating a serious and widespread impact of LF. According to the CDC, it is estimated that 100,000 to 300,000 cases of LF occur per year in the endemic regions of West Africa, and it causes approximately 5000 deaths per year among identified cases. With the exception of Dengue fever, the estimated global burden of LF is the highest among all viral hemorrhagic fevers [5], and in 2016, the World Health Organization identified LASV as a top priority emerging pathogen and recommended accelerated vaccine development [6].

In the early stage of infection, LF is often misdiagnosed with other tropical febrile illnesses due to the presence of non-specific signs and symptoms, such as fever, headache, arthralgia, myalgia, and asthenia, which are observed 6 to 12 days after infectious contact. Pharyngitis, conjunctivitis, cough, abdominal pain, facial or neck edema, diarrhea, vomiting, and abnormal bleeding can appear at later disease stages. Severely affected patients die because of hypotensive, hypovolemic, and hypoxic shock, whereas the symptoms disappear within 10 to 15 days after disease onset in surviving patients [7–9]. The observation that about 4 out of 5 patients successfully control LASV infection and recover

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Research in context

Evidence before this study

Lassa virus (LASV) is responsible of devastating hemorrhagic illnesses that occurs in West Africa. For instance, the crosstalk between the virus and the cellular components of the innate immune system during acute infection has yet to be deciphered, LASV infection seems to be associated with defective immune responses.

Added value of this study

Findings from this study indicate a specific association between the *KIR2DL2* gene and fatalities in a cohort of LASV⁺ patients from Sierra Leone, in association with specific epitopes of the nucleoprotein and envelope glycoprotein from LASV when presented by HLA-C molecules

Implications of all the available evidence

These data provide novel insights into the mechanisms by which LASV can escape NK-cell-mediated immune pressure.

suggests that LF can induce effective immunity, whereas severe LASV infection could be associated with defective immune responses and even virus-induced immunosuppression.

Natural killer (NK) cells are an important component of the antiviral immune response. They have the ability to lyse target cells and to regulate adaptive immune responses through the production of cocktails of chemokines and cytokines [10]. NK cell activity is determined by the integration of inhibitory and activating signals that arise from the binding of a vast array of receptors present on the surface of these cells. Such receptors include the killer-cell immunoglobulin-like receptors (KIRs): KIR2DL1, KIR2DL2, and KIR3DL1, which bind human leukocyte antigen (HLA) class-1 of the C1, -C2, and -Bw4 groups, respectively, and result in the inhibition of NK-cell function [11–13]. Furthermore, genetic studies have uncovered associations between specific KIR:HLA class-1 combinations and particular outcomes to various viral infections, including Ebola virus infection [14–16].

LASV exhibits broad tissue tropism, with dendritic cells (DC) being important in vivo targets of infection [17]. In vitro, LASV-stimulated NK cells acquire an enhanced cytolytic potential against K562 target cells that lack HLA class-1 molecules, whereas LASV-infected DC with constitutive HLA class-1 molecule expression remain resistant to NK cell lysis [18]. It was hypothesized that a viral sequence within HLA class-1-presented epitopes might lead to enhanced engagement of inhibitory KIRs expressed on NK cells inhibiting NK cell activity against infected cells. KIRs recognize their cognate HLA class-1 ligands in a peptide-dependent manner, as emphasized by the resolution of crystal structures of KIR/peptide/HLA complexes [19]. Amino-acid point mutations within viral peptides might allow for viral escape from NK-cell-mediated immune recognition, as reported in HIV-1 [13,20,21]. However, the mechanism by which LASV can enable evasion from antiviral NK cells remains poorly understood. Therefore, this study aimed to determine the impact of inhibitory KIRs on NK cells and their cognate HLA class-1 ligands on the susceptibility to LASV infection.

2. Materials and methods

2.1. Study subjects

In total, 37 survivors and 14 non-survivors LASV-infected patients were included in this study. They are compared to healthy controls

from the same geographical origin in Kenema, Sierra Leone ($n = 68$), and in Franceville, Gabon ($n = 54$) [22]. This study was conducted in accordance with the principles of the Declaration of Helsinki and with French statutory and regulatory law. Patients received information about research performed on biological samples and provided written informed consent to participate. This study was approved by the Sierra Leone Ethics and Scientific Review Committee, and the French institutional ethic committee (Comité de Protection des Personnes of Ile de France VI).

2.2. KIR and HLA genotyping

DNA was extracted from whole blood using QIAamp DNA blood mini kit (Qiagen) from laboratory-confirmed LASV survivors and fatalities in Kenema Government hospital (Kenema, Sierra Leone). KIR genotyping was performed by polymerase chain reaction (PCR) using standard primers and internal controls, as previously described [23]. HLA class-1 alleles were hybridized using the LABType SSO kit (One Lambda) following the manufacturer's instructions.

2.3. Cells and antibodies

The class-1 MHC-negative human B cell line 721.221 (transduced with the TAP inhibitor ICP47) [24] was transfected with HLA-B*53:01, HLA-B*27:05, HLA-C*05:01, HLA-C*06:02, HLA-C*07:01, or HLA-C*08:02 (kindly provided by Prof. J. Strominger, Harvard University, USA) using the Amaxa nucleofactor kit (Lonza). Surface expression of HLA molecules on 721.221-ICP47-transfected cells was analyzed by flow cytometry using the pan-anti-HLA class-1 monoclonal antibody (#W6/32, Thermo-Fisher Scientific) as previously described [25].

NK-cell lines were obtained from 3 characterized healthy donors: NK-NAM: 2DL1⁻2DL2⁺2DL3⁻3DL1⁺; NK-DEP: 2DL1⁻2DL2⁻2DL3⁺3DL1⁺, and NK-RIM: 2DL1⁺2DL2⁻2DL3⁺3DL1⁻ [26]. NK cells were purified by using magnetic-activated cell sorting (MACS) separation columns (Miltenyi Biotec) and cultured in the presence of 100 U/mL recombinant IL-2 (Proleukin-2; Prometheus), as previously described [27]. NK cells were analyzed with KIR2DL1-Fc, KIR2DL2-Fc, or KIR3DL1-Fc fusion proteins (R&D Systems) followed by a secondary staining with a goat anti-human Fc-specific fragment-phycoerythrin antibody (Thermo-Fisher Scientific), as described [21].

2.4. Epitope prediction tools

Consensus sequence and polymorphisms were obtained by comparison of the entire 210 LASV glycoprotein (GP) and nucleocapsid protein (NP) sequences listed on NCBI resources (<https://www.ncbi.nlm.nih.gov>), and were analyzed using the Weblogo software (<http://weblogo.berkeley.edu/>) (Supplementary Fig. 1).

To screen for potential HLA-C1, HLA-C2, and HLA-Bw4 binding epitopes among LASV GPs and NPs, the entire consensus sequences were entered into the NetMHCpan 4 HLArestrictor tool program (<http://www.cbs.dtu.dk/services/HLArestrictor/>). Standard recommended threshold settings were used to screen and select peptides that potentially led to the strongest interaction with HLA-C1 (*03:03, 07:01, 07:02, 08:02, 12:03, and 14:02), HLA-C2 (*04:01, 05:01, 06:02, and 15:02), and HLA-Bw4 (*0802, 0803, 2705, 3801, 4402, 4403, 5101, 5103, 5701, and 5801) and their naturally occurring variants. The negative NP₂₃₀ "IDTKKSSLNS" control peptide was selected due to its inability to bind any of the HLA class-1 molecules (percent rank between 55% and 99%, depending on HLA class-1 alleles). In addition, a scramble peptide from the GP₁₉₂ LASV peptide (Scr/GP₁₉₂; AWMGYSYRMG) as well as two HLA-C*06:02 restricted positive control peptides: the MUM-2 derived peptide, FRSGLDYSV [28,29], and the RunX3 derived peptide, ARFNDLRFV [30] are used in some experiments. Each peptide was then validated experimentally. All selected 10-mer peptides were synthesized by Covalabs (Villerban, France).

Table 1
KIR genotypes and HLA ligand combinations in survivors and fatalities of LASV infections, compared to healthy populations from Gabon and Sierra Leone.

	Ctl Gabon (n = 54)		Ctl SL (n = 68)		LASV positive (n = 51)			Survivors (n = 37)			Fatalities (n = 14)		
	n	%	n	%	n	%	p	n	%	p	n	%	p
Inhibitory KIR genotype													
2DL1	54/54	100	68/68	100	51/51	100	ns	37/37	100	ns	14/14	100	ns
2DL2	35/54	64.8	50/68	73.5	42/51	82.3	ns	28/37	75.7	ns	14/14	100	0.032
2DL3	43/54	79.6	54/68	79.4	45/51	88.2	ns	30/37	94.6	ns	10/14	71.4	ns
2DL5	39/54	72.2	30/68	44.1	20/51	39.2	ns	15/37	40.5	ns	5/14	50.0	ns
3DL1	54/54	100	67/68	98.5	50/51	98.0	ns	36/37	97.3	ns	14/14	100	ns
3DL2	54/54	100	68/68	100	51/51	100	ns	37/37	100	ns	14/14	100	ns
3DL3	54/54	100	68/68	100	51/51	100	ns	37/37	100	ns	14/14	100	ns
Activating KIR genotype													
2DL4	54/54	100	68/68	100	51/51	100	ns	37/37	100	ns	14/14	100	ns
2DS1	10/54	18.5	14/68	20.6	8/51	15.7	ns	5/37	13.5	ns	3/14	21.4	ns
2DS2	31/54	57.4	23/68	33.8	19/51	37.2	ns	14/37	37.8	ns	5/14	35.7	ns
2DS3	19/54	35.2	24/68	35.3	23/51	45.1	ns	15/37	40.5	ns	8/14	57.1	ns
2DS4	54/54	100	68/68	100	51/51	100	ns	37/37	100	ns	14/14	100	ns
2DS5	18/54	33.3	21/68	66.2	13/51	25.5	ns	11/37	29.7	ns	2/14	14.3	ns
3DS1	4/54	7.4	6/68	8.8	2/51	3.9	ns	1/37	2.7	ns	1/14	7.1	ns
Inhibitory KIR:HLA associations													
C2 ⁺ in 2DL1 ⁺	34/47	68.0	30/37	81.1	27/34	79.4	ns	19/23	82.6	ns	8/11	72.7	ns
C1 ⁺ in 2DL2 ⁺	20/31	64.5	19/29	65.5	22/30	73.3	ns	12/19	63.1	ns	10/11	90.1	ns
C1 ⁺ in 2DL3 ⁺	27/38	71.0	25/31	80.6	22/33	66.6	ns	15/21	71.4	ns	7/11	71.4	ns
Bw4 ⁺ in 3DL1 ⁺	33/47	70.2	24/37	64.9	10/34	29.4	ns	6/23	26.1	ns	4/11	36.4	ns
Activating KIR:HLA associations													
C2 ⁺ in 2DS1 ⁺	7/8	87.5	5/6	83.3	nd	nd	nd	3/4	75.0	ns	nd	nd	nd
C1 ⁺ in 2DS2 ⁺	18/22	64.3	8/13	61.5	7/12	58.3	ns	5/9	55.5	ns	2/3	66.7	ns

Ct Gabon: Healthy controls from Gabon, West Africa (Petitdemange PLoS ONE 2014); Ctl SL: Healthy controls from Sierra Leone, West Africa; P: Fisher exact test between studied group and Ctl SL. ns: non-significant.

2.5. Peptide-HLA stabilization and KIR binding assay

HLA-transfected 721.221-ICP47 cells were pulsed with 100 μM of the indicated selected peptide for 24 h, as described [21]. After staining with the anti-pan-HLA class-1 monoclonal antibody (#W6/32), cells were analyzed using a Navios flow cytometer (Coulter). As controls, cells were cultured in the absence of peptide or pulsed with the negative NP₂₃₀ peptide. Titration experiments were performed using 100 μM, 10 μM, or 1 μM peptide.

KIR binding assays were performed after 24 h peptide-HLA stabilization. Briefly, peptide-pulsed 721.221-ICP47 cells were stained with adequate KIR-Fc fusion protein [21]. After staining, target cells were analyzed by flow cytometry.

2.6. Polyfunctional assay

NK cell lines from the 3 characterized healthy donors were incubated with peptide-pulse HLA-transfected 721.221-ICP47 cells at an effector:target (E:T) cell ratio of 1:1, in the presence of anti-CD107a mAb (#H4A3; Becton Dickinson) to measure degranulation. Cells were thereafter incubated for 5 h in the presence of Golgi Stop and Golgi Plug solutions (BD Biosciences) and then stained with cell-surface markers (anti-CD3, anti-CD56, and anti-CD45). Cells were fixed, permeabilized with a cytofix/cytoperm kit (Becton Dickinson), and then intracellularly stained with anti-IFN-γ (#B27; Becton Dickinson) and anti-TNF-α (#Mab11; eBiosciences), as previously described [31]. Data were analyzed with the “Boolean gate” algorithm using Flow Jo version 9 (TreeStar). Pestle software was used to remove the background, and pie charts, generated with Spice software (NIAI freeware) [32], present the frequency of NK cells positive for 0, 1, 2, or 3 responses. Arcs depict the frequency of cells positive for CD107a, IFN-γ, or TNF-α.

2.7. Statistical analysis

Statistical analyses were performed with Prism software (GraphPad, CA, USA). The non-parametric Kruskal-Wallis tests were used as appropriate for comparison of continuous variables between groups.

3. Results

3.1. Association of HLA class-1 and inhibitory KIR genotype with fatal outcome in LASV Infection

We compared individual KIR genotypes of 68 healthy volunteers to 51 patients infected with LASV, including 37 survivors and 14 fatalities. Percentages of KIR gene carriers in the Sierra Leone and Gabonese control samples were similar to each other (Table 1) and to other African cohorts [22,33]. Overall, LASV-infected patients were similar to healthy controls, except for the inhibitory KIR2DL2 gene. While survivors (28/37, 75.7%) were equivalent to controls (50/68; 73.5%), 100% of fatal cases carried the KIR2DL2 gene ($p = .032$) (Table 1).

Since the interactions between KIR and their ligands are essential to control NK-cell function, we next evaluated the frequency of KIR genes in combination with the genes that encoded their respective known ligands in the same patients. We observed a similar proportion of HLA-Bw4 allele among KIR3DL1⁺ survivor, fatal and control patients (Table 1) and it was also the case for the genetic combination of KIR2DL1 and HLA-C2 between infected and healthy samples. In contrast, we noticed a specific increase in the HLA-C1:KIR2DL2 interaction in fatalities (10/11; 90.1%) as compared to survivors (12/19; 63.1%) and control individuals (19/29; 65.5%). While the data did not reach statistical significance (due to the small proportion of fatalities in LASV) they suggested that HLA-C1:KIR2DL2 interaction could certainly participate to the fatal outcome LASV infection.

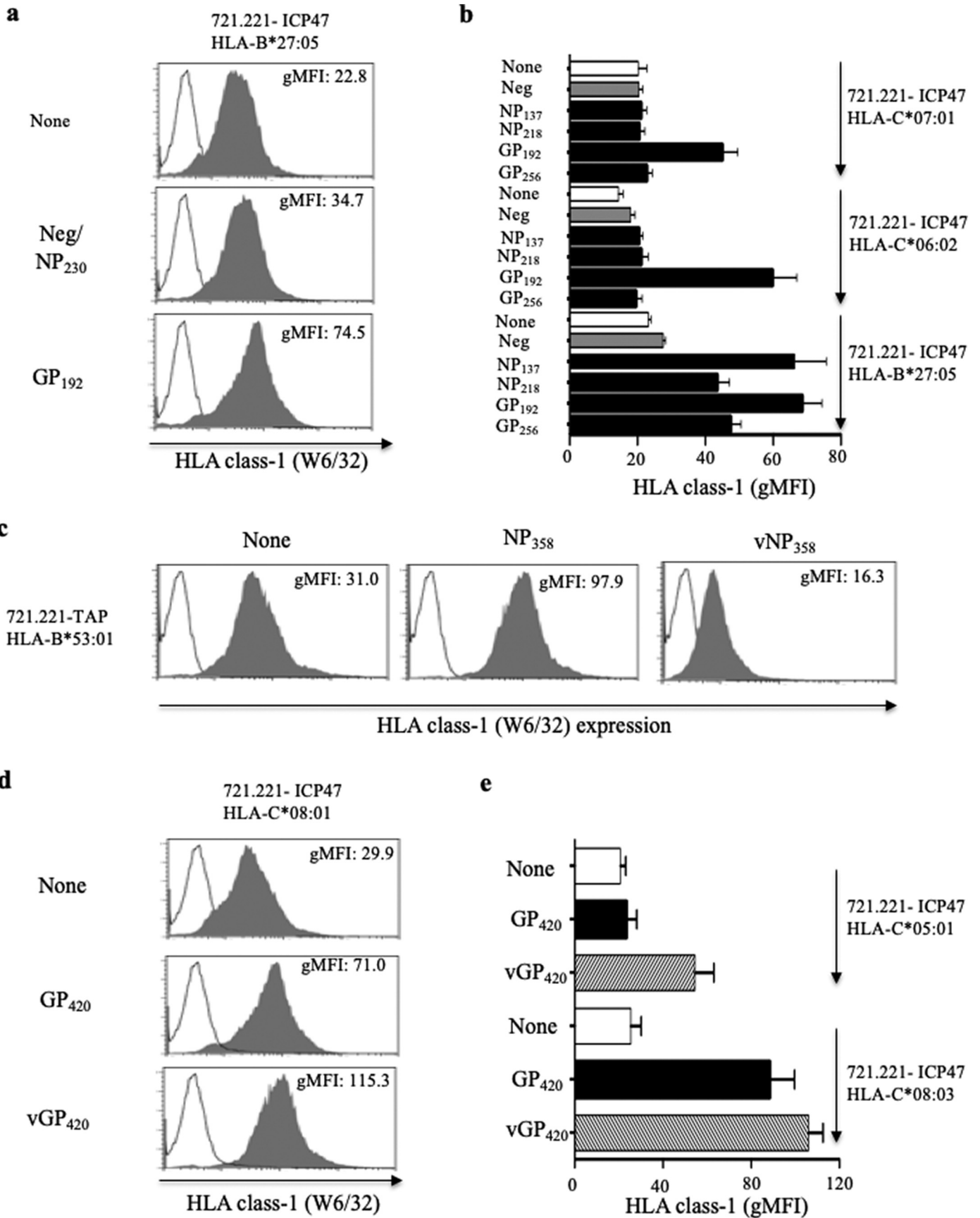


Table 2
Selection of naturally occurring variants of HLA optimal epitopes for NU and GP LASV proteins.

Sequences	Frequency N (%)	HLA-C1		HLA-C2		HLA-Bw4		
		*C07:01	C*08:02	C*06:02	C*05:01	B*27:05	B*53:01	
NP ₃₅₈	FPTGLTYSQL	118 (56.2)	47	15	60	21	37	0.12
	FTAGLTYSQL	71 (33.8)	27	8	35	14	19	5
GP ₄₂₀	YMERQGKTPL	86 (40.9)	25	0.5	33	16	12	21
	YIDRQGKTPL	59 (28.1)	29	0.1	36	1.5	25	21

Data are expressed in NetMHCpan percentile rank score; the lower score is associated with the higher predicted affinity. Frequency is based on the 210 NP and GP analyzed sequences. Point-mutations are underlined.

3.2. Identification of KIR/HLA class-1 associated epitopes within GP and NP LASV proteins

We next investigated whether peptides from GP and NP LASV proteins presented by HLA class-1 molecules on target cells can impact NK cell function through HLA-C1:KIR2DL2 interaction. We initially obtained consensus sequences from the 210 published entire sequences of NP and GP from LASV (Supplementary Fig. 1). These consensus sequences were used to identify potential epitopes using the NetMHCpan 4 HLAREstrictor predictive tool (<http://www.cbs.dtu.dk/services/HLAREstrictor/>). A similar number of weak and strong interactions were found with NP and GP viral proteins. Most of these interactions were linked to HLA-Bw4, but not HLA-C (C1 and C2) molecules, and reached statistical significance for GP (Fisher test: $p = .0309$; Odds ratio: 0.0898, IC 95%: [0.0016; 1.0191]) (Supplementary Table 1). To experimentally confirm these data, we utilized 721.221 cells transfected with ICP47 which blocks transporter associated with antigen processing (TAP) function and pulsed these cells with different HLA class-1 molecules (B*27:05, B*53:01, C*05:01, C*06:02, C*07:01 or C*0802) to determine the ability of specific peptides to bind to and stabilize HLA class-1 molecules. Fig. 1a shows that pulsing with wild-type peptides sharply induces expression of HLA class-1 molecules, as detected with the W6/32 (HLA-A,B,C) monoclonal antibody. Similar data were obtained with other tested LASV peptides but only with regard to their specific cognate HLA class-1 molecules (data not shown). Of note, the low binding activity in the absence of peptide could suggest that self-peptides can be loaded, in previously described [21,34]. Unexpectedly, GP₁₉₂ was the sole epitope able to promote multiple strong interactions with HLA-C1 (C*07:01), HLA-C2 (C*06:02), and HLA-Bw4 (B*27:05 and B*38:01) molecules (Fig. 1a-b; Supplementary Table 2). As controls, strong binding was also observed with MUM-2 and RunX3 derived peptides, two HLA-C*06:02 restricted positive control peptides, whereas lower binding was observed in the presence of a scramble peptide from GP₁₉₂ (Supplementary Fig. 2a).

Previous studies in HIV-1 and other infections demonstrated that viral escape resulted from immune pressure by selection of sequence polymorphisms [20]. Considering that main anchor positions are typically composed of hydrophobic amino acid residues in position 2 and the C-terminus [35], we identified only a few polymorphisms in NP (vNP₃₅₈) and GP (vGP₁₃₂, vGP₃₇₄, vGP₄₂₀ and vGP₄₇₁) sequences that led to stronger HLA class-1 interactions compared to their wildtype counterparts (Supplementary Table 2). Most of these variants, however, were detected in less than 5% of the sequences, although vNP₃₅₈ and vGP₄₂₀ were observed in 33.8% and 28.1% of sequences, respectively (Table 2; Supplementary Table 2). We confirmed that vNP₃₅₈ was associated with a loss of interaction with HLA-B*53:01 (Fig. 1c), whereas

vGP₄₂₀ increased binding to HLA-C1 (C*08:02) and promoted binding to HLA-C2 (C*05:01), compared to wild-type peptides (Fig. 1d-e). Titration experiments using serial peptide dilutions, confirmed equal HLA stabilization induced by GP₄₂₀ wild-type and variant peptides (Supplementary Fig. 3).

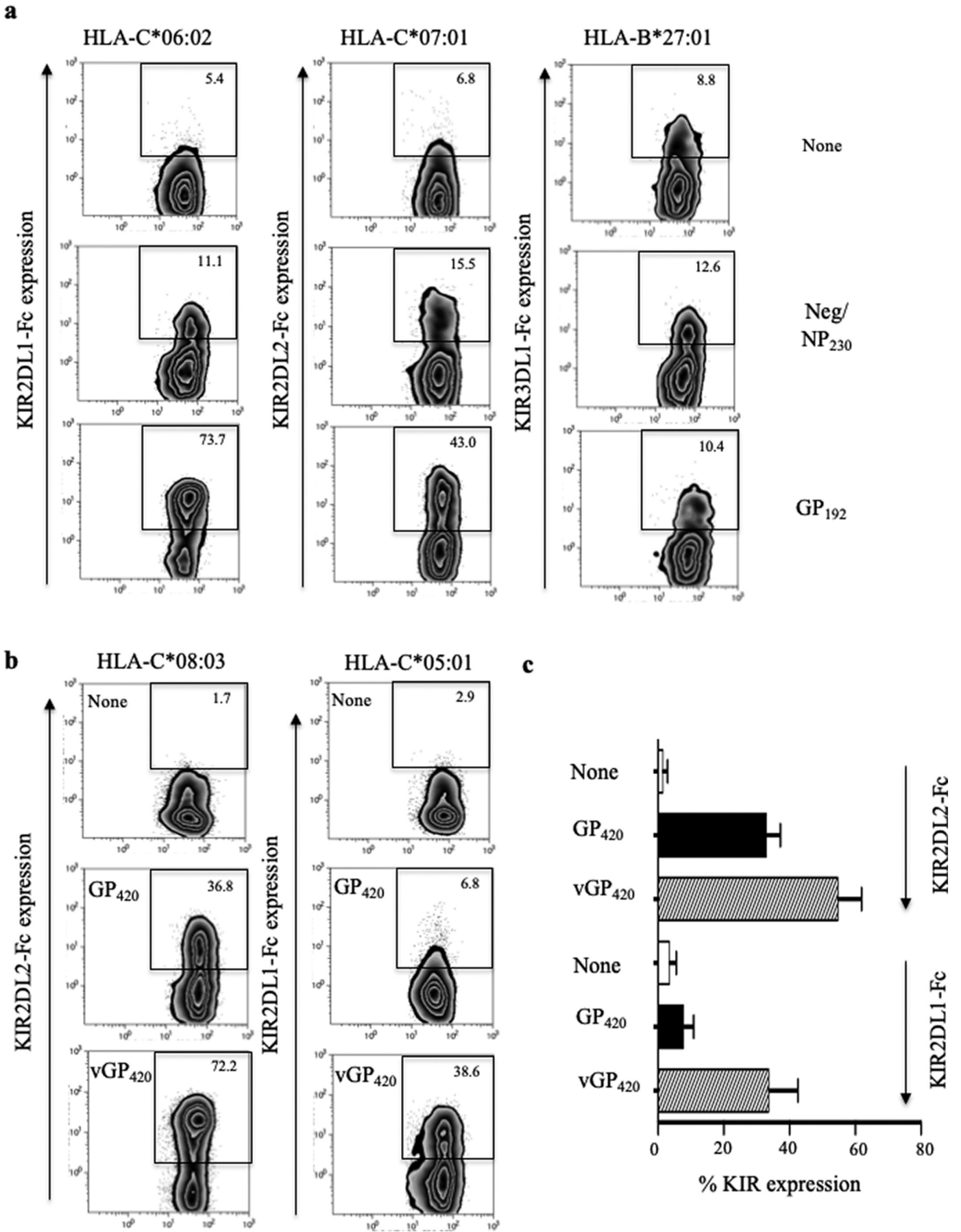
3.3. LASV epitopes from NP and GP enhanced binding to inhibitory KIR2DL receptors

To assess the consequences of NP and GP epitope presentation, KIR/HLA interactions were assessed in the presence of specific KIR-Fc fusion proteins. Strong KIR-Fc binding was detected with all tested LASV peptides in the presence of HLA-C1 and -C2 molecules (Fig. 2a; Supplementary Fig. 3a). In addition, GP₄₂₀ wild-type and variant peptides bound similarly to HLA-C1 and -C2 molecules in the presence of KIR2DL1-Fc and KIR2DL2-Fc proteins, respectively (Fig. 2b). The results of repeated binding assays are shown in Fig. 2c. Unexpectedly, no specific KIR3DL1-Fc binding was observed in the presence of peptides that led to strong interaction with the different HLA-Bw4 molecules, such as HLA-B*27:01 (Fig. 2a). Similar data were obtained with NP₃₅₈ wild-type and variant peptides in the presence of 721.221-ICP47-HLA-B*53:01 cells (Supplementary Fig. 4a-4b), and with other tested peptides (data not shown). As controls, strong interaction with KIR2DL1 was also observed with MUM-2 and RunX3 derived peptides, two HLA-C*06:02 restricted positive control peptides, whereas very low interaction was detected in the presence of a scramble peptide from GP₁₉₂ (Supplementary Fig. 2b).

3.4. KIR/HLA-C-associated LASV epitopes specifically inhibits NK-cell function

We next investigated whether strong KIR2DL-Fc binding to HLA-C induced by LASV epitopes could modulate the effector function of specific KIR⁺ NK cells. Primary NK cells from different KIR/HLA-typed healthy donors were co-incubated with HLA-C-transfected 721.221-ICP47 cells pulsed with selected peptides. NK cell function was assessed to simultaneously detect degranulation by CD107a/LAMP1 cell-surface expression and intracellular cytokine production (interferon gamma [IFN- γ] and tumor necrosis factor alpha [TNF- α]). In the absence or presence of a control peptide that did not allow for KIR binding, target cells strongly induced both degranulation and cytokine production (Fig. 3a). In contrast, while the NP and GP LASV peptides led to strong HLA-C1 and -C2 interactions, they primarily inhibited degranulation in the presence of KIR2DL2⁺ and KIR2DL1⁺ NK cells, respectively (Fig. 3a-b). As controls, very low level of degranulation of KIR2DL1⁺ NK cells was also observed with MUM-2 and RunX3 derived peptides,

Fig. 1. Stabilization of HLA Class-1-Transfected 721.221-ICP47 Cells Pulsed with NP and GP LASV Peptides. (a) Representative histograms of HLA-B*27:07 stabilization without peptide (None), with negative NP₂₃₀ control peptide, or with GP₁₉₂ peptide. Peptides were added at a final concentration of 100 μ M. Surface level expression of HLA class-1 molecules was determined by flow cytometry using the anti-pan HLA class-1 W6/32 mAb. (b) Quantification of different HLA class-1 stabilization (HLA-C*07:01, C*06:03, and B*27:05) (N = 5) in the absence (None) or in the presence of negative NP₂₃₀ control peptide, or of selected peptides (NP₂₁₈, GP₁₉₂, and GP₂₅₆). Data represents the mean of 5 experiments and error bars indicate standard deviation. (c) Representative histograms of HLA-B*53:03 stabilization without peptide (None) or with wild-type NP₃₅₈ and vNP₃₅₈ variant peptides. (d-e) Representative histograms and quantification of 5 HLA-C*08:01 stabilizations without peptide (None) or with wildtype GP₄₂₀ and vGP₄₂₀ variant peptides. Cells were then stained with isotype control (single line) or with W6/32 monoclonal antibody (solid grey line). Data are expressed as the geometric mean of fluorescence (gMFI).



two HLA-C*06:02 restricted positive control peptides, at two E/T ratio (1/1 and 5/1), whereas high degranulation was observed in the presence of a scramble peptide from GP₁₉₂, closed to the baseline observed with untreated cells (Supplementary Fig. 2c). Similar results were obtained in the presence of NK cells that expressed KIR2DL3 (data not shown). Furthermore, this inhibitory effect significantly increased in the presence of the vGP₄₂₀ variant peptide, compared to wild-type, and degranulation was altered for 721.221-ICP47 cells transfected with HLA-C*05:01 ($p = .081$) and C*08:03 ($p = .019$) (Fig. 3c-d).

Notably and in accordance with the KIR3DL1-Fc binding assay (Supplementary Fig. 4a-4b), the addition of NP or GP LASV peptides that binds different HLA-Bw4 alleles did not alter NK cell degranulation or cytokine production whether the NK cells expressed KIR3DL1 (Fig. 3a, right panels; Supplementary Fig. 5) or not (data not shown). Overall, our results suggest that inhibition of NK cell degranulation and cytokine production is restricted to interaction of HLA-C molecules pulsed with specific LASV peptides and inhibitory KIR2DL2/DL3 receptors.

4. Discussion

Prompted by observations that NK cells can induce immune selection pressure in several viral infections, we explored specific KIR-HLA interactions in LASV-infected patients and then investigated the impact of LASV-derived epitopes on primary NK-cell function. We observed that *KIR2DL2* expression was closely associated with a poor diagnosis in LF and identified several HLA-C binding epitopes in LASV proteins that can inhibit NK-cell function.

We performed a genetic comparison of KIRs and their cognate HLA-class-1 ligands between healthy individuals and LF patients from the Sierra Leone population. We did not observe strong associations except for the inhibitory *KIR2DL2* gene, where a significantly increased frequency was exclusively observed in the fatalities group. In contrast, we have shown previously that in patients infected by Ebola virus from Zaire (ZEBOV), another hemorrhagic fever, all inhibitory KIRs were unchanged, whereas the activating *KIR2DS1* and *KIR2DS3* genes were considerably more prevalent among non-survivors [16]. These discrepancies could be related to differences in the physiopathological process of LASV infection, which is associated with an inhibition of NK-cell function, whereas ZEBOV infection is characterized by an excessive activation of the immune response that is responsible for a rapid depletion in NK cells and other lymphocytes [2]. These observations suggest that different KIR combinations could provide a permissive immune microenvironment that fosters a fatal outcome. However, we cannot exclude that our results were influenced by population demographic history requiring a confirmation through large-scale studies in patients with different hemorrhagic fevers and in other ethnic groups.

In LASV infection, *KIR2DL2* could participate in the inhibition of NK-cell function in the presence of HLA class-1⁺ infected target cells [18]. Our results indicate that several epitopes from NP and GP LASV proteins led to strong interactions with different HLA-B and HLA-C class-1 molecules. These findings are consistent with previous studies that demonstrated that self-epitopes as well as peptides derived from EBV, HCV, and HIV-1 presented by HLA class-1 molecules impact NK-cell function via specific inhibitory KIRs [36–38]. Currently, our knowledge of the peptide-binding specificity of HLA class-1 molecules differs considerably among the different isotypes. There are more than 50,000 examples of HLA-B interactions that represent the different allotypes and less than 1000 instances registered for HLA-C molecules in the immune

epitope database (www.iedb.org). Accordingly, we found significantly more LASV epitopes that strongly interacted with HLA-Bw4 as compared to HLA-C molecules. However, it is important to note that all tested peptides that interact with HLA-Bw4 molecules did not result in detectable binding to KIR3DL1. In contrast, peptide:HLA-C interactions were associated with strong binding with their respective inhibitory KIR receptor, and this interaction significantly inhibited NK cell function. It is however important to note that immunogenetic analysis of KIR and HLA, as well as LASV epitope inhibition assays suggest that interaction of HLA-C1 with KIR2DL2 and KIR2DL3 could have two opposite consequences, facilitation and protection, respectively, whereas these two KIR have more than 98% homology in their ligand binding domains. To confirm this hypothesis these analyses should be analyzed in a larger cohort of LASV-infected patients. These data are consistent with previous studies on functional inhibition of NK cells observed after co-culture of LASV-infected human DC and NK cells, as well as in LASV-infected macaques [17,39]. Taken together, our results elucidate a mechanism by which LASV can evade NK-cell-mediated immune pressure through HLA-C-presented epitopes in order to inhibit the function of NK cells. In this context, the absence of functional effect after interaction between LASV peptides and HLA-B molecules may suggest that Bw4 epitopes could be more involved in binding to the T-cell receptor (TCR) on T cells than on NK cells. Conversely, HLA-C has been thought to play a less important role relative to HLA-A or -B in defense against viral infection via T cell immunity, since HLA-C molecules are expressed at a lower level on their cell surface [40–42].

Several studies have reported that peptide sequence variations may enhance the binding of inhibitory KIRs to the peptide/HLA complexes and, consequently, down-regulate the activity of NK cells [13,21,43–45]. We also observed naturally occurring variants in NP and GP LASV peptides that led to strong interactions with HLA class-1 molecules. However, these variants were very rare in comparison to chronic infections, like HIV-1, where variants continuously evolve to maintain a viral escape from immune control [13,21]. In LASV, the vG₄₂₀ sequence variation is frequently selected in acute infections (28.1%) and associated with stronger binding to the inhibitory KIR2DL2 receptor. This interaction is in line with genetic evidence that supports a role for *KIR2DL2* in LASV infection. The stronger inhibition of the NK cell function could suggest that *KIR2DL2*⁺ NK cells exert immune pressure on the virus to select for viral peptides that provide a stronger antiviral response, as previously shown in virus-infected mice [46]. Taken together, our data provide a mechanism by which LASV might be able to evade NK-cell-mediated recognition through the presentation of viral epitopes that engage specific inhibitory KIR receptors.

Author contributions

N.W., C.P., P.D. and V.V. contributed to the project design and prepared the ethical protocols. N.W., M.C., V.L., and J.B. processed samples at Government Kenema Hospital; N.W. provided samples for the experiments; N.W., C.P., N.T., C.M., and V.V. performed experiments; V.V. directed the research and wrote the manuscript.

Declaration of interests

N.W. has been a consultant to Metabiota. Metabiota had no role in study design, data analysis, data interpretation, or writing of the manuscript. The other authors declare no competing interests.

Fig. 2. Binding of KIR-Fc by HLA Class-1-Transfected 721.221-ICP47 Cells Pulsed with NP and GP LASV Peptides. (a) Representative dot plots of KIR-Fc staining of HLA class-1-transfected 721.221-ICP47 cells pulsed without peptide (None), with negative NP₂₃₀ control peptide, or GP₁₉₂ peptide. Data are shown with HLA class-1-transfected 721.221-ICP47 cells transfected with HLA-C*06:02, C*07:01, and B*27:01, and stained with KIR2DL1-Fc, KIR2DL2-Fc, or KIR3DL1-Fc, respectively. (b-c) Representative dot plots and quantification of KIR2DL2-Fc and KIR2DL1-Fc staining ($N = 5$) from 721.221-ICP47 cells transfected HLA-C*08:03 or C*05:01, respectively, and pulsed without peptide (None) or with wild-type GP₄₂₀ and vGP₄₂₀ variant peptides.

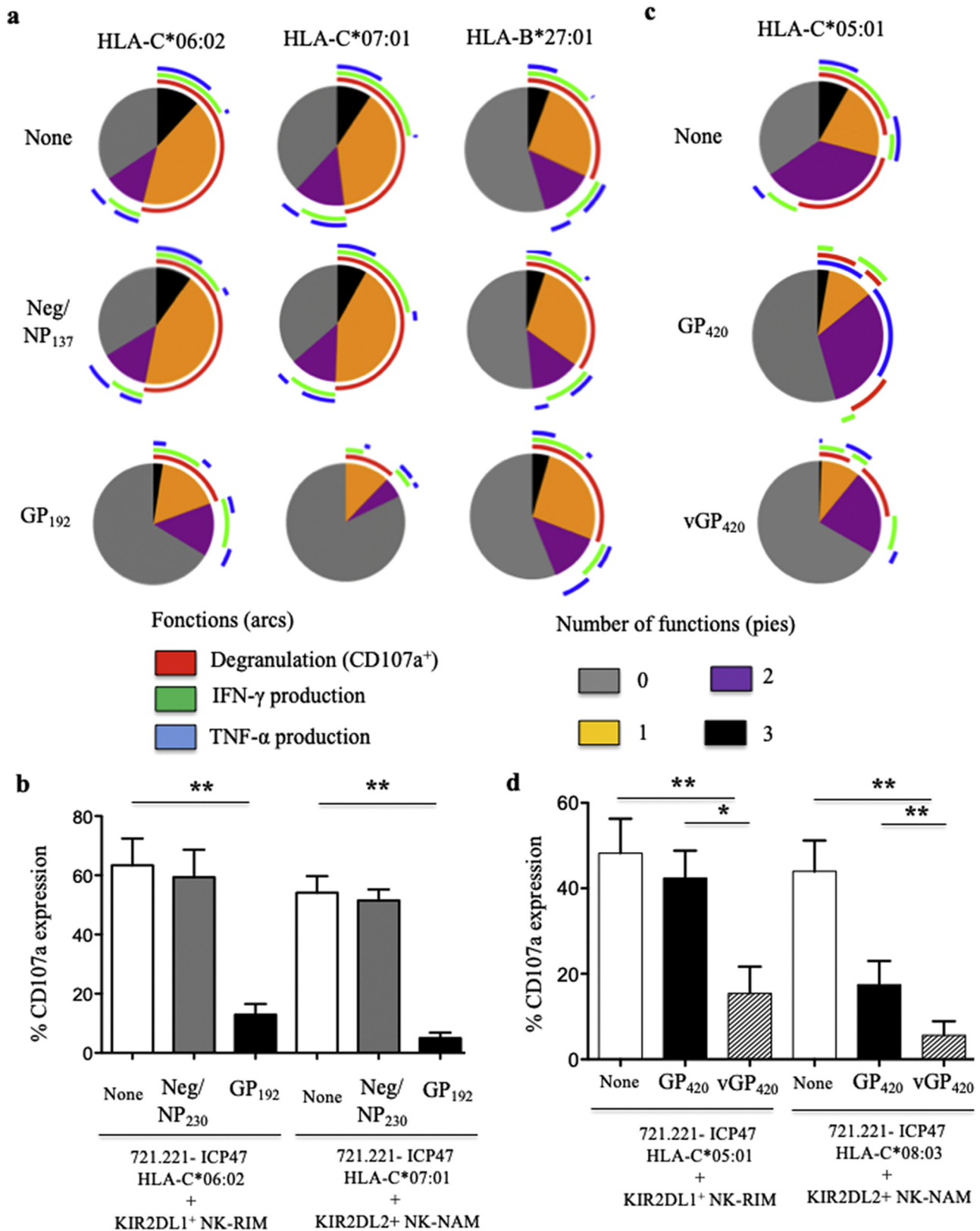


Fig. 3. Functional NK cell activity in the presence of HLA class-1-transfected 721.221-ICP47 cells pulsed with NP and GP LASV peptides. (a) Representative polyfunctional assay (degranulation and cytokine production) of KIR2DL2⁺ NK-NAM cell line, in the presence of HLA class-1-transfected 721.221-ICP47 cells pulsed without peptide (None), with negative NP₂₃₀ control peptide, or with GP₁₉₂ peptide. Polyfunctionality was determined using SPICE and PESTLE software (NIH). Single, double or triple cytokine producing cells are color coded in pies as indicated in the Pie slice legend. Pie chart arcs represent overlapping production of cytokines and are color coded as indicated in the corresponding legend. (b) Quantification of CD107a frequency ($n = 5$) in selected KIR⁺ NK cell lines in the presence of HLA-C-transfected 721.221-ICP47 cells pulsed without peptide (None), with negative NP₂₃₀ control peptide, or with GP₁₉₂ peptide. (c) Representative polyfunctional assay of KIR2DL1⁺ NK-RIM cell line in the presence of HLA-C*05:01-transfected 721.221-ICP47 cells pulsed without peptide (None) or with wild-type GP₄₂₀ and vGP₄₂₀ variant peptides. (d) Quantification of CD107a frequency ($n = 5$) in select KIR⁺ NK cell lines in the presence of HLA-C transfected-721.221-ICP47 cells pulsed without peptide (None) or with wild-type GP₄₂₀ and vGP₄₂₀ variant peptides.

Acknowledgments

We are grateful to Joseph Fair and Jean Paul Gonzales from Metabiota. We thank Solenne Chardonnet (CIMI-Paris) for technical help. These studies are funded by the National Agency of Research (ANR) under reference ANR-13-BSV-0004. N.W. and C.P. have received fellowships from ANR. N.W. and C.P. were in part supported by the ANR (ANR-13-BSV-0004).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.01.048>.

References

- [1] McCormick JB, Webb PA, Krebs JW, Johnson KM, Smith ES. A prospective study of the epidemiology and ecology of Lassa fever. *J Inf Dis* 1987;155(3):437–44.
- [2] Prescott JB, Marzi A, Safronetz D, Robertson SJ, Feldmann H, Best SM. Immunobiology of Ebola and Lassa virus infections. *Nat Rev Immunol* 2017;17(3):195–207.
- [3] Fisher-Hoch SP, Tomori O, Nasidi A, Perez-Oronoz GI, Fakile Y, Hutwagner L, et al. Review of cases of nosocomial Lassa fever in Nigeria: the high price of poor medical practice. *BMJ* 1995;311(7009):857–85.
- [4] Ogbu O, Ajuluchukwu E, Eneke CJ. Lassa fever in West African sub-region: an overview. *J Vector Borne Dis* 2007;44(1):1–11.
- [5] Falzarano D, Feldmann H. Vaccines for viral hemorrhagic fevers—progress and shortcomings. *Curr Opin Virol* 2013;3(3):343–51.
- [6] WHO. Annual Review of Diseases Prioritized Under the Research and Development Blueprint; 2017 (Geneva, Switzerland. <http://www.who.int/blueprint/priority-diseases/en>).
- [7] Schoepp RJ, Rossi CA, Khan SH, Goba A, Fair JN. Undiagnosed acute viral febrile illnesses, Sierra Leone. *Emerg Infect Dis* 2014;20(7):1176–82.
- [8] O’Hearn AE, Voorhees MA, Fetterer DP, Wauquier N, Coomber MR, Bangura J, et al. Serosurveillance of viral pathogens circulating in West Africa. *Virol J* 2016;13(1):163.
- [9] Raabe V, Koehler J. Laboratory diagnosis of Lassa fever. *J Clin Microbiol* 2017;55(6):1629–37.
- [10] Lam VC, Lanier LL. NK cells in host responses to viral infections. *Curr Opin Immunol* 2017;44:43–51.
- [11] Lanier LL. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol* 2008;8(4):259–68.
- [12] Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science* 2011;331(6013):44–9.
- [13] Saunders PM, Vivian JP, O’Connor GM, Sullivan LC, Pymm P, Rossjohn J, et al. A bird’s eye view of NK cell receptor interactions with their MHC class I ligands. *Immunol Rev* 2015;267(1):148–66.
- [14] Rajagopalan S, Long EO. Understanding how combinations of HLA and KIR genes influence disease. *J Exp Med* 2005;201(7):1025–9.
- [15] Kulkarni S, Martin MP, Carrington M. The Yin and Yang of HLA and KIR in human disease. *Semin Immunol* 2008;20(6):343–52.
- [16] Wauquier N, Padilla C, Becquart P, Leroy E, Vieillard V. Association of KIR2DS1 and KIR2DS3 with fatal outcome in Ebola virus infection. *Immunogenetics* 2010;62(11–12):767–71.
- [17] Russier M, Pannetier D, Baize S. Immune responses and Lassa virus infection. *Viruses* 2012;4(11):2766–85.
- [18] Russier M, Reynard S, Tordo N, Baize S. NK cells are strongly activated by Lassa and Mopeia virus-infected human macrophages in vitro but do not mediate virus suppression. *Eur J Immunol* 2012;42(7):1822–32.
- [19] Stewart-Jones GB, di Gleria K, Kollnberger S, McMichael AJ, Jones EY, Bowness P. Crystal structures and KIR3DL1 recognition of three immunodominant viral peptides complexed to HLA-B*2705. *Eur J Immunol* 2005;35(2):341–51.
- [20] Alter G, Heckerman D, Schneidewind A, Fadda L, Kadie CM, Carlson JM, et al. HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature* 2011;476(7358):96–100.
- [21] Hölzemer A, Thobakgale CF, Jimenez Cruz CA, Garcia-Beltran WF, Carlson JM, van Teijlingen NH, et al. Selection of an HLA-C*03:04-restricted HIV-1 p24 gag sequence variant is associated with viral escape from KIR2DL3+ natural killer cells: data from an observational cohort in South Africa. *PLoS Med* 2015;12(11):e1001900.
- [22] Petitdemange C, Wauquier N, Jacquet JM, Theodorou I, Leroy E, Vieillard V. Association of HLA class-I and inhibitory KIR genotypes in Gabonese patients infected by Chikungunya or Dengue type-2 viruses. *PLoS One* 2014;9(9):e108798.
- [23] Vilches C, Castaño J, Gómez-Lozano N, Estefanía E. Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments. *Tissue Antigens* 2007;70(5):415–22.
- [24] Gatfield J, Lammert E, Nickolaus P, Münz C, Rothenfusser S, Fisch P, et al. Cell lines transfected with the TAP inhibitor ICP47 allow testing peptide binding to a variety of HLA class I molecules. *Int Immunol* 1998;10(11):1665–72.
- [25] Mandelboim O, Reyburn HT, Sheu EG, Vales-Gomez M, Davis DM, Pazmany L, et al. The binding site of NK receptors on HLA-C molecules. *Immunity* 1997;6(3):341–50.
- [26] Nguyen S, Achour A, Souchet L, Vigouroux S, Chevallier P, Furst S, et al. Clinical impact of NK-cell reconstitution after reduced intensity conditioned unrelated cord blood transplantation in patients with acute myeloid leukemia: analysis of a prospective phase II multicenter trial on behalf of the Société Française de Greffe de Moelle Osseuse et Thérapie Cellulaire and Eurocord. *Bone Marrow Transplant* 2017;52(10):1428–35.
- [27] Baychelier F, Sennepin A, Ermonval M, Dorgham K, Debré P, Vieillard V. Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. *Blood* 2013;122(17):2935–42.
- [28] Dionne SO, Lake DF, Grimes WJ, Smith MH. Identification of HLA-Cw6.02 and HLA-Cw7.01 allele-specific binding motifs by screening synthetic peptide libraries. *Immunogenetics* 2004;56(6):391–8.
- [29] Chapel A, Garcia-Beltran WF, Hölzemer A, Ziegler M, Lunemann S, Martrus G, et al. Peptide-specific engagement of the activating NK cell receptor KIR2DS1. *Sci Rep* 2017;7(1):2414.
- [30] Mobbs JL, Illing PT, Dudek NL, Brooks AG, Baker DG, Purcell AW, et al. The molecular basis for peptide repertoire selection in the human leucocyte antigen (HLA) C*06:02 molecule. *J Biol Chem* 2017;292(42):17203–15.
- [31] Petitdemange C, Becquart P, Wauquier N, Béziat V, Debré P, Leroy EM, et al. Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. *PLoS Pathog* 2011;7(9):e1002268.
- [32] Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* 2011;79(2):167–74.
- [33] Denis L, Sivula J, Gourraud PA, Kerdudou N, Chout R, Ricard C, et al. Genetic diversity of KIR natural killer cell markers in populations from France, Guadeloupe, Finland, Senegal and Réunion. *Tissue Antigens* 2005;66(4):267–76.
- [34] Sim MJ, Malaker SA, Khan A, Stowell JM, Shabanowitz J, Peterson ME, et al. Canonical and cross-reactive binding of NK CELL INHIBITORY RECEPTORS to HLA-C allotypes is dictated by peptides bound to HLA-C. *Front Immunol* 2017;8:193.
- [35] Falk K, Röttschke O, Stevanović S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991;351(6324):290–6.
- [36] Das J, Khakoo SI. NK cells: tuned by peptide? *Immunol Rev* 2015;267(1):214–27.
- [37] Lin Z, Kuroki K, Kuse N, Sun X, Akahoshi T, Qi Y, et al. HIV-1 control by NK cells via reduced interaction between KIR2DL2 and HLA-C*12:02/C*14:03. *Cell Rep* 2016;17(9):2210–20.
- [38] Lunemann S, Martrus G, Hölzemer A, Chapel A, Ziegler M, Körner C, et al. Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C*03:04 and modulate NK cell function. *J Hepatol* 2016;65(2):252–8.
- [39] Baize S, Marianneau P, Loth P, Reynard S, Journeaux A, Chevallier M, et al. Early and strong immune responses are associated with control of viral replication and recovery in Lassa virus-infected cynomolgus monkeys. *J Virol* 2009;83(11):5890–903.
- [40] Mandelboim O, Reyburn HT, Valés-Gómez M, Pazmany L, Colonna M, Borsellino G, et al. Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules. *J Exp Med* 1996;184(3):913–22.
- [41] Apps R, Qi Y, Carlson JM, Chen H, Gao X, Thomas R, et al. Influence of HLA-C expression level on HIV control. *Science* 2013;340(6128):87–91.
- [42] Rasmussen M, Harndahl M, Stryhn A, Boucherma R, Nielsen LL, Lemonnier FA, et al. Uncovering the peptide-binding specificities of HLA-C: a general strategy to determine the specificity of any MHC class I molecule. *J Immunol* 2014;193(10):4790–802.
- [43] Thananchai H, Makadzange T, Maenaka K, Kuroki K, Peng Y, Conlon C, et al. Reciprocal recognition of an HLA-Cw4-restricted HIV-1 gp120 epitope by CD8+ T cells and NK cells. *AIDS* 2009;23(2):189–93.
- [44] Fadda L, Körner C, Kumar S, van Teijlingen NH, Piechocka-Trocha A, Carrington M, et al. HLA-Cw*0102-restricted HIV-1 p24 epitope variants can modulate the binding of the inhibitory KIR2DL2 receptor and primary NK cell function. *PLoS Pathog* 2012;8(7):e1002805.
- [45] van Teijlingen NH, Hölzemer A, Körner C, García-Beltrán WF, Schafer JL, Fadda L, et al. Sequence variations in HIV-1 p24 Gag-derived epitopes can alter binding of KIR2DL2 to HLA-C*03:04 and modulate primary natural killer cell function. *AIDS* 2014;28(10):1399–408.
- [46] Tu MM, Mahmoud AB, Makrigrannis AP. Licensed and unlicensed NK cells: differential roles in cancer and viral control. *Front Immunol* 2016;7:166.