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CD8+ T lymphocytes are sensitive to NKG2A/HLA-E licensing interaction: role in the survival of cancer patients

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ABSTRACT

NK and CD8$^+$ T cells are the main cytolytic effectors involved in innate and adaptive tumor immune surveillance, respectively. Although their educational pathways differ, similarities in their development and function suggest that CD8$^+$ T lymphocytes could be sensitive to NK cell licensing signals, which might influence their antitumor response. To demonstrate this hypothesis, we retrospectively evaluated the impact that NK cell licensing interactions have on the expression of CD226 on CD8$^+$ T lymphocytes and on the survival of patients with different hematopoietic and solid cancers (n = 1,023). Prospectively, we analyzed by multiparametric flow cytometry the anti-CD3/CD28-induced proliferation and immune-receptor expression of purified CD8$^+$ T lymphocytes from healthy donors (n = 17) with different combinations of NK cell licensing ligands. Results show that methionine/threonine (M/T) dimorphism at position −21 of the HLA-B leader peptide, but not other HLA class-I dimorphisms involved in the education of NK cells (HLA-C1/C2 or HLA-Bw4), is associated with greater survival and expression of CD226 in cancer patients, which was proportional to the number of methionines present in their genotype. CD8$^+$ T lymphocytes from healthy donors with −21 M showed higher proliferation rates and lower expression of TIGIT after $in\ vitro$ stimulation. Therefore, CD8$^+$ T lymphocytes, like NK cells, appear to be sensitive to the −21 M/T dimorphism of HLA-B leader peptide, which results in the modulation of CD226 $in\ vivo$ and the proliferation and expression of TIGIT after $in\ vitro$ stimulation, all of which could be related to their immune-surveillance capacity and the survival of cancer patients.

Introduction

Multiple factors are involved in the survival of cancer patients after receiving adequate risk-adapted therapies. Among them, genetic alterations of cancer that determine aggressiveness and treatment sensitivity stand out, but also the genetic diversity of the patient's immune system can determine the quality and quantity of immune cell interactions with the tumor cell. In recent years, our group has demonstrated the relevance of key receptor/ligand interactions that regulate the education and function of NK cells to the survival of patients with myeloma,[1] childhood acute leukemia,[2] melanoma, bladder and ovarian cancers.[3–5] However, the phenotypic, developmental and functional similarities of NK and CD8$^+$ T cells[6] suggest that CD8$^+$ T lymphocytes could also be sensitive to interactions regulating NK cell education and function.

Although the interaction of CD8$^+$ T lymphocytes with tumor cells relies on the T-cell receptor (TCR), which recognizes specific peptides presented in human leukocyte antigen class-I (HLA-I), and NK/tumor cell interaction relies on

multiple preformed activating and inhibiting receptors, which recognize conserved regions of HLA-I, both entrust their education, self-toleranceand acquisition of functional competence to the recognition of HLA-I molecules.[6–8] While CD8$^+$ T-cell education at initial developmental stages takes place in the thymus through very well-known positive and negative selection processes,[9] NK cell maturation and acquisition of functional competence is a continuous process that occurs in the bone marrow and the periphery through the recognition of their own HLA-I by NKG2A and inhibitory killer-cell Immunoglobuline-like receptors (iKIR) in a process called "licensing".[10–12] Licensing allows NK cells to discriminate healthy tissues from tissues expressing damage/danger signs, loss of HLA-I ligands for inhibitory receptors ("missing-self")[13,14] or even alterations in the peptidome presented by HLA-I ("altered-self").[15,16]

Inhibitory receptor involvement in the functional education of NK cells was first reported for mice and humans in 1998 and 2006, respectively,[10,17] but clear phenotypic and metabolic

evidence was reported in humans in 2015[18] and 2018,[19] respectively. NK cell education through inhibitory receptors is strongly linked to increased expression of DNAM-1 (CD226) and to conformational changes in LFA-1 (CD11a),[18,20] both contributing to stabilize NK/tumor cell synapse and with this tumor cell lysis.[18] Furthermore, licensed NK cells show enhanced glycolysis to support their increased proliferative and cytotoxic capacities[19] and lysosomal remodeling with accumulation of granzyme B in dense-core secretory lysosomes.[21] Although the clinical implications of these interactions have been poorly studied, alterations in CD226 expression lead to higher relapse rates in AML[22] and higher susceptibility to solid and hematologic cancers.[23–26]

Licensing interactions include, on the one hand, the highly diversified interactions of KIR with HLA-I such as KIR2DL1/C2-epitope (HLA-C allotypes with Lys80 in alpha-1 helix); KIR2DL2-3/C1-epitope (HLA-C allotypes with Asn80 and the rare allotypes HLA-B*46:01 and HLA-B*73:01),[15,27] although with lower affinity, KIR2DL2-3 can also interact with C2-epitope;[15] KIR3DL1/Bw4-epitope of HLA-A and HLA-B allotypes (either with threonine or isoleucine 80);[28] KIR3DL2/HLA-A*03 and A*11 allotypes;[29] and, on the other hand, the conserved interaction of CD94:NKG2A with HLA-E.[30] The binding site of HLA-E is specific for nonamer peptides corresponding to residues −22 to −14 of the leader sequence of HLA-A, -B and -C,[31,32] with a dimorphism in the anchor residue at position −21. Methionine −21 (−21 M), present in all HLA-A and -C allotypes and in a minority of HLA-B allotypes, provides a good anchor residue that facilitates the folding and cell-surface expression of HLA-E.[33] In contrast, threonine −21 (−21 T), the residue present in the majority of HLA-B allotypes, does not bind effectively to HLA-E. Nonetheless, HLA-C is poorly expressed on the cell surface[34] and HLA-A peptides are rarely used to restrict CD8+ T-cell epitopes;[35,36] thus, HLA-B leader peptides seem to have a dominant contribution to generate HLA-E binding peptides.[37] Indeed, HLA-B peptide dimorphism at this position has been associated with susceptibility to HIV infection,[38] killing of HIV-infected cells by NK cells,[39] and more recently, with NK cell anti-leukemic activity and overall survival of acute myeloid leukemia patients under IL-2 immunotherapy.[40]

Genetic analysis of human populations worldwide shows how haplotypes with −21 M HLA-B rarely encode for the Bw4 or C2 KIR ligands;[30,41] therefore, there are two fundamental forms of HLA haplotype: one preferentially supplying NKG2A ligands and the other supplying KIR ligands. Thus, dimorphism at position −21 of the leader sequence of HLA-B divides the human population into MM, MTand TT groups. Functional assays have shown that individuals with −21 M haplotypes have NKG2A+ NK cells that are better educated, phenotypically more diverseand functionally more potent than those of TT individuals.[30] This study investigates whether the signals mediated by KIR/HLA-I or NKG2A/HLA-E interactions could also modulate the tumor immune surveillance function of CD8+ T lymphocytes.

Patients and methods

Samples and study groups

This study included 23,586 healthy volunteers (control group) and 1,023 consecutive patients with different types of cancer: plasma cell neoplasm ($n = 322$), melanoma ($n = 311$), bladder cancer ($n = 193$), pediatric acute leukemia ($n = 97$), ovarian cancer ($n = 88$) and other tumors ($n = 12$). An institutional review board (IRB-00005712) approved the study. Written informed consent was obtained from all patients and controls in accordance with the Declaration of Helsinki. Detailed information on biological, clinical and evolutionary features of patients is summarized in Table 1.

Peripheral blood samples anticoagulated with EDTA were obtained both from all controls and from patients for HLA-I (KIR ligands) and KIR genotyping. Additionally, immunophenotype analysis was performed in 582 patients with cancer to evaluate the expression of activating (CD16, CD226 and aKIR) and inhibitory (iKIR and NKG2A) receptors on NK and T cells (Supplementary Figure 1). For in vitro stimulation of purified CD8+ T cell, peripheral blood samples anticoagulated with sodium heparine were obtained from 17 healthy volunteers with different genotypes at position −21 for the leader peptide of HLA-B (MM, MT or TT).

Table 1. Biological, clinical and evolutionary features of patients.

	n	Age (Years)	Sex (male)	Follow-up (months)	Progression (rate)	Dead (rate)
Solid Cancer[a]	528					
Melanoma	311	60.2 ± 15.5	53.8%	60.3 ± 45.1	35.1%	9.4%
Bladder	193	71.1 ± 10.1	85.3%	28.3 ± 27.1	34.6%	20.0%
Ovarian	88	58.7 ± 10.7	0.00%	18.8 ± 21.9	42.0%	22.7%
Plasma cell neoplasm[a]	322					
MGUS	160	67.3 ± 11.5	53.1%	61.6 ± 29.8	55.0%	13.5%
Multiple myeloma	162	67.1 ± 12.8	52.3%	54.9 ± 37.6	45.0%	37.8%
Childhood acute leukemia[a]	97					
B Lymphoblastic	70	8.3 ± 4.9	60.0%	41.9 ± 32.9	2.8%	10.0%
T lymphoblastic	16	9.2 ± 4.5	75.0%	46.6 ± 30.6	12.5%	0.00%
Myeloid	16	7.7 ± 5.6	56.3%	37.7 ± 25.9	31.3%	31.3%

[a]Detailed information on the histological characteristics for each type of cancer can be found in previous publications in which these patients were included.[1–5]
MGUS: monoclonal gammopathy of undetermined significance.

Figure 1. Immunophenotype analysis after *in vitro* stimulation with anti-CD3/CD28. (a) Gaiting strategy consisted in the following steps: selecting total lymphocytes in an FSC/SSC dot plot, selecting singlets in an FSC-A/FSC-H dot plot, selecting 7-AAD negative and positive alive and dead cells in a dot plot 7-AAD/NKG2D, and selecting CD8^{+} T cells in a CD8/CFSE dot plot and then logical gating to identify singlet alive and dead CD8^{+} T cells. Histogram gates were set in CFSE to identify cells in the zero, first, second, third, fourthor fifth proliferation cycle for alive cells, and in the zero or >1 cycle for dead cells. (b) Dot plots showing CFSE staining in the FITC channel versus the other 11 fluorescences with CD8-BV605, CD226-PE, CD25-PECy7, CD27-APC, CD38-AF700, NKG2D-APCCy7), TIGIT-BV421, CD45RA-BV510, TIM-3-BV711 and NKG2A-BV786.

HLA-A, -B and -C and KIR genotyping

KIR and HLA-A, -B, and -C genotyping was performed in DNA samples extracted from peripheral blood with QIAmpDNABlood Mini kit (QIAgen, GmbH, Hilden, Germany) using sequence-specific oligonucleotide PCR (PCR-SSO) and Luminex® technology with Lifecodes KIR-SSO (cat. 545,110 R) and LifecodesHLA-SSO HLA-A (cat. 628,913), HLA-B (cat. 628,917) and HLA-C (cat. 628,921) typing kits (Immucor Transplant Diagnostic, Inc. Stamford, CT, USA), as previously described.[4,20] HLA-A and HLA-B genotyping allowed us to identify alleles bearing the Bw4 motif according to the amino-acid sequences at positions 77–83 in the α1 domain of the HLA class-I heavy chain. Bw4 alleles with threonine at amino acid 80 (80 T) and higher affinity for KIR3DL1 (HLA-B*05, B*13, B*44) were distinguished from those with isoleucine 80 (I80) and lower affinity (HLA-A*23, A*24, A*25, A*32 and HLA-B*17, B*27, B*37, B*38, B*47, B*49, B*51, B*52, B*53, B*57, B*58, B*59, B*63 and B*77). HLA-C genotyping allowed distinction between HLA-C alleles with asparagine 80 (C1-epitope: HLA-C*01, 03, 07, 08, 12, 14, 16:01) and lysine 80 (C2-epitope: HLA-C*02, *04, *05, *06, *15, *16:02, *17, *18).[27] Nonetheless, the KIR ligand calculator at https://www.ebi.ac.uk/ipd/kir/ligand.html was used to ascertain Bw4, C1 and C2 epitopes. Dimorphism at position −21 of the leader sequence of HLA-B was also discriminated to distinguish allotypes with methionine (−21 M, HLA-B*07, B*08, B*14, B*38, B*39, B*42, B*48, B*67, B*73 and B*81) from those with threonine (−21 T, rest HLA-B alleles).

KIR genotyping identified iKIRs (KIR2DL1, KIR2DL2, KIR2DL3, KRIR2DL5, KIR3DL1, KIR3DL2, and KIR3DL3) and activating KIRs (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, and KIR3DS1), as well as KIR2DL4, which exhibits both inhibitory and activating potential. The method used could not distinguish between KIR2DL5A (telomeric) and KIR2DL5B (centromeric) forms. Different allotypes of KIR2DS4 were detected, including the expressed allotype KIR2DS4 full-length exon-5 (KIR2DS4f, KIR2DS4*001-002 alleles) and the non-expressed KIR2DS4 deleted exon-5 (KIR2DS4d, KIR2DS4*003,*004, *006, *007, *008 alleles).

Flow cytometry immunophenotype analysis of cancer patients

The expression of CD226 (DNAM-1), NKG2A, and KIR receptors (KIR2DL1, 2DS1, 2DL2/S2, 2DL3 and 3DL1) on both CD3⁻CD16⁻/⁺CD56⁺⁺ (CD56^{bright}) and CD3⁻CD16⁺CD56⁺ (CD56^{dim}) NK cell subsets and CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells was evaluated as a percentage of positive cells, and as mean fluorescence intensity (MFI) using LSR-II and DIVA Software (BD), as previously described.[4,20] Photomultiplier (PMT) voltages were adjusted daily using rainbow calibration particles (Biolegend). Fluorescence compensations were adjusted every 2 months using blood samples stained for simple fluorochromes with conjugated anti-CD4 antibodies for each cytometer detector and then re-adjusted finely during the analysis using negative events as reference for each fluorochrome. The staining protocol included 11-color/12-monoclonal antibody (mAb): CD3 AmCyam (clone SK7, BD), CD4 PE-CF594 (RPA-T4, BD), CD8 APCCy7 (SK1, BD), CD16 PacBlue (3G8, BD), CD56 BV711 (NCAM16.2, BD), CD158a,h PECy7 (EB6, BD, recognizes both KIR2DL1 and 2DS1), CD158b1/b2,j PE-Cy5 (GL183, Beckman-Coulter, recognizes KIR2DL2, 2DL3 and 2DS2), CD158a FITC (143,211, R&D systems, KIR2DL1), CD158b2 APC (180,701, R&D systems, KIR2DL3), CD158e APC (DX9, R&D systems, KIR3DL1), CD226 PE (11A8, Biolegend), and NKG2A biotin (REA110, Miltenyi Biotech). Streptavidin AF700 (Life Technologies, molecular probes) was used to detect biotinylated NKG2A.

Supplementary Figure 1 shows the gating strategy used for the analysis. Briefly, an FSC/SSC dot plot was used to identify lymphocytes; a CD3/CD16 dot plot to select CD3⁺ T cells; a CD4/CD8 dot plot to identify principal T-cell subsets; a CD16/CD56 dot plot to identify CD56^{bright} and CD56^{dim} NKcs; a CD56/CD226 dot plot to select CD226⁺ cells; a CD3/NKG2A dot plot to identify NKG2A⁺ cells; a KIR2DL1S1/KIR2DL1 dot plot to distinguish KIR2DL1⁺, KIR2DS1⁺, double-positive KIR2DL1/S1, and double-negative KIR2DL1/S1 NKcs; and a KIR2DL2/S2/L3/KIR3DL1 dot plot to distinguish KIR2DL2S2⁺, KIR2DL3⁺, KIR3DL1⁺, and triple-negative KIR2DL2S2/L3/3DL1 NK cells. Internal negative cells were used as isotype controls to set positive cutoffs. Gates were hierarchically and electronically combined to define the following NK cell subsets: (1) CD56^{bright}; (2) CD56^{dim} KIR⁻NKG2A⁺ NKcs; (3) CD56^{dim} KIR⁻NKG2A⁻ (non-licensed); and (4) CD56^{dim} NK cells single-positive for KIR2DL1, 2DS1, 2DL2/S2, 2DL3, or 3DL1. Gates were adapted according to the expression of these molecules in each patient.

Anti-CD3/CD28 stimulation in vitro of purified CD8⁺ T cells

Purified CD8⁺ T lymphocytes were stimulated in vitro in triplicate with anti-CD3/CD28 coated streptabidin-dynabeads as described elsewhere.[42,43] Briefly, 20 ml of sodium heparin anticoagulated peripheral blood samples were obtained from healthy donors with TT (n = 7), MT (n = 7) or MM (n = 3) genotypes at position −21 of the leader sequence of HLA-B. CD8⁺ T lymphocytes were enriched by negative selection in ficoll density gradients using RosetteSep® Human CD8⁺ T-cell kit (Stemcell Technologies, Grenoble, France) and stained with 0.05 μM carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific, Waltham, MA). CFSE-labeled CD8⁺ T cells (1 x 10^5/well) were then stimulated at 37°C in a 5% CO₂ incubator, in 96-well U-bottomed plates, with three beads per cell of magnetic streptabidin-dynabeads (Thermo Fisher Scientific) coated with 0.1 μg/ml of biotinylated anti-CD3 and 0.1 μg/ml of biotinylated anti-CD28 (1 μg of antibody for 0.1 mg of beads). After 96 hours, the harvested cells were stained with CD226-PE (Biolegend, Clon 11A8), 7-AAD (Invotrogen, Merck Life Science, S.L.U.), CD25-PECy7 (BD, Clon 2A3), CD27-APC (BD, Clon L128), CD38-AF700 (BD, Clon HIT2), NKG2D-APCCy7 (Biolegend, Clon 1D11), TIGIT-BV421 (BD, Clon 741,182), CD45RA-BV510 (BD, Clon HI100), CD8-BV605 (BD, Clon SK1), TIM-3-BV711 (BD, Clon 7D3) and NKG2A-BV786 (BD, Clon 131,411), and 5 x 10^4 cells acquired in a 12 fluorescence FACSLyric (BD). Analysis strategy is shown in Figure 1.
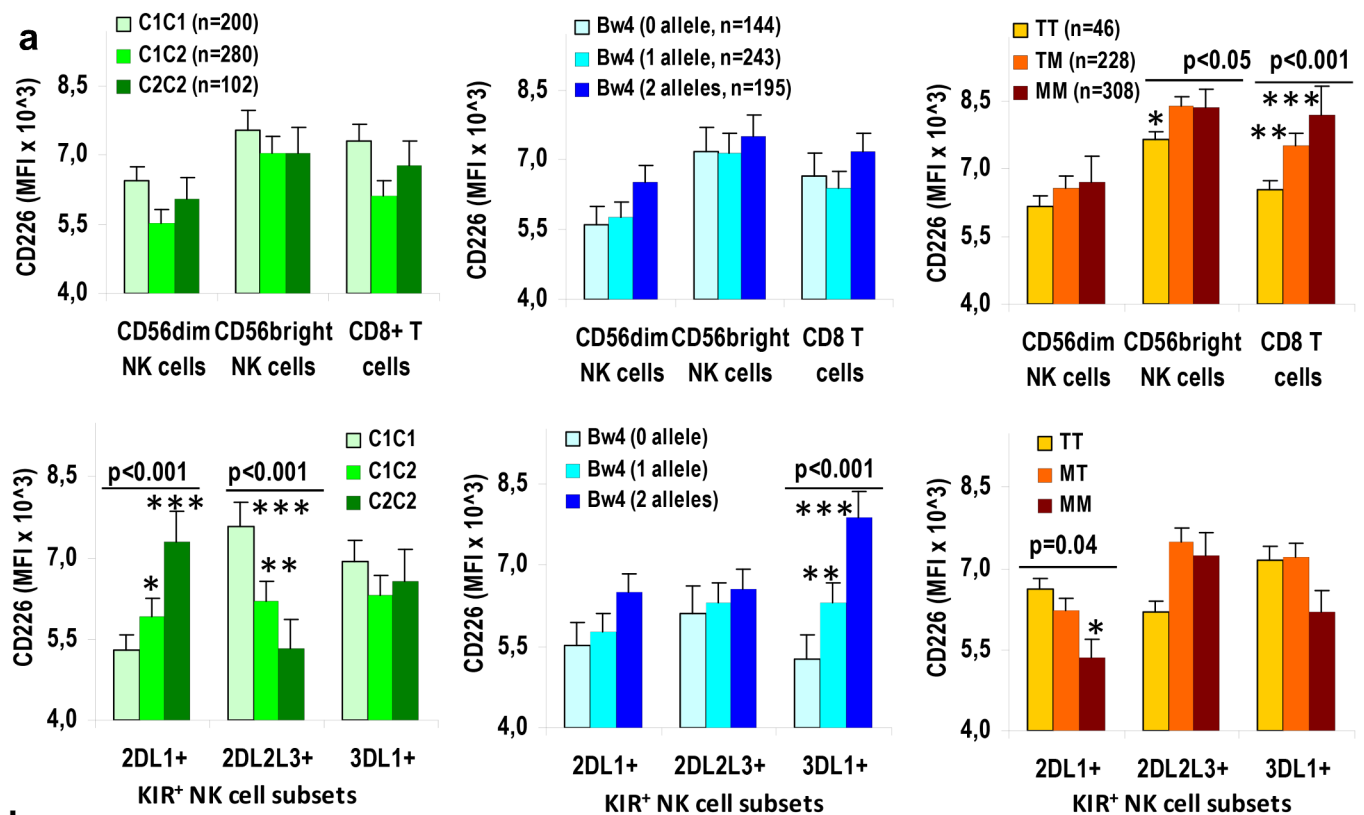
Statistical analysis

All data were collected in a database (Excel 2003; Microsoft Corporation, Seattle, WA) and analyzed using SPSS version 15.0 (SPSS Inc., Chicago, IL). Pearson's χ2 or two-tailed Fisher's exact tests were used to analyze categorical variables. Student's t or ANOVA with *post hoc* tests were used to analyze continuous variables. Kaplan–Meier estimator and log-rank tests were used to analyze patient overall survival (OS). Time to death was estimated as months from the diagnosis date. Multivariate analysis for patient OS was performed using the Cox proportional hazards model (stepwise regression). The hazard ratio (HR) and 95% confidence interval were estimated. Data are expressed as mean ± SEM. P values < .05 were considered significant.

Results

CD226 expression is up-modulated on NK and T cells in presence of specific inhibitory-receptor/ligand interactions

CD226 up-modulation is the main, and basically the only, sign to identify licensed NK cells in peripheral blood.[18,20] For that purpose, the expression of CD226 was evaluated in NK and T-cell subsets in cancer patients according to the presence of HLA-I specific ligands. CD226 expression did not show differences in NK CD56^{dim}, NK CD56^{bright} or CD8^{+} T cells in the presence of C1, C2 or Bw4 HLA-ligands. However, the presence of −21 M was associated with increased expression of CD226 in NK CD56^{bright} ($p < .05$) but, more significantly, in CD8^{+} T cells ($p < .001$), where the CD226 increase was directly proportional to the copy number of −21 M HLA-B alleles (Figure 2a, upper

Figure 2. CD226 expression is up-modulated on NK and T cells in presence of specific inhibitory-receptor/ligand interactions. (a) Mean fluorescence intensity (MFI) of CD226 in CD56^{dim} and CD56^{bright} NK cells and CD8^{+} T cells (upper plots) and in KIR2DL1^{+}, KIR2DL2/L3^{+} and KIR3DL1^{+} NK cell subsets (lower plots) in presence of HLA C1, C2 or Bw4 ligands and −21 M/T dimorphism. P values indicate global estimation in the ANOVA test; * $p < .05$, ** $p < .01$, and *** $p < .001$ in the *post hoc* tests for each group. (b) Contingency tables for C1, C2 and Bw4 HLA-I ligands versus −21 M/T dimorphism in controls and cancer patients. P values estimated in the χ2 test.

Table for Figure 2b:

| | \multicolumn{5}{c|}{Healthy controls (n=23,586)} | \multicolumn{5}{c}{Cancer patients (n=1,023)} |
	N	TT (n=13,574)	MT (n=8,604)	MM (n=1,408)	p	N	TT (n=573)	MT (n=379)	MM (n=71)	p
C1C1	8,446	38.1%	46.7%	15.2%	<0.001	342	34.0%	46.6%	19.4%	<0.001
C1C2	10,038	59.1%	39.3%	1.60%		473	59.6%	39.6%	0.80%	
C2C2	5,102	85.4%	14.6%	0%		208	88.5%	10.8%	0%	
Bw4 (0 allele)	5,051	34.8%	48.9%	16.3%	<0.001	250	33.0%	47.8%	19.1%	<0.001
Bw4 (1 allele)	11,612	57.0%	38.7%	4.30%		489	55.8%	38.9%	5.30%	
Bw4 (2 allele)	6,923	65.5%	32.5%	2.10%		284	72.3%	24.5%	3.30%	

bar-plot). In CD4 + T cells, this −21 M-related CD226 increase was not observed (data not shown). However, the M/T dimorphism did not influence the frequency of TCD4+, TCD8+ or NK cell subsets expressing the CD226 molecule.

NK cell subsets with inhibitory KIR receptors did show very significant increases in the expression of CD226 in the presence of their specific ligands. Thus, C2, C1and Bw4 HLA-ligands induced a dose-dependent increase in the expression of CD226 on KIR2DL1[+], KIR2DL2/L3[+] and KIR3DL1^{+} NK cells ($p < .001$), respectively. In contrast, −21 M only modulated negatively the expression of CD226 in KIR2DL1^{+} NK cells ($p = .04$) (Figure 2a, lower bar-plot). However, this was mostly due to the inverse association of C2 HLA-ligands with −21 M allotypes observed in patients and controls in our series (Figure 2b), as previously described.[30,41]

Methionine −21 of the HLA-B leader peptide is associated with longer survival in patients with different types of cancer

Next, we explored the impact that HLA-B M/T −21 dimorphism had on the OS of patients. Although no significant results were observed in the log-rank test, a clear trend toward better OS was observed in a −21 M dose-dependent way for all cancer patients and for each type of cancer including solid cancer (melanoma, bladder and ovarian carcinomas), plasma cell neoplasm and childhood acute leukemia (Figure 3). In addition, HLA-B M/T −21 dimorphism ($p = .04$) together with the patient´s age ($p = .002$) were the only independent predictive factors for patients' OS when analyzed with C1, C2, and Bw4 HLA-ligands and the type of cancer.
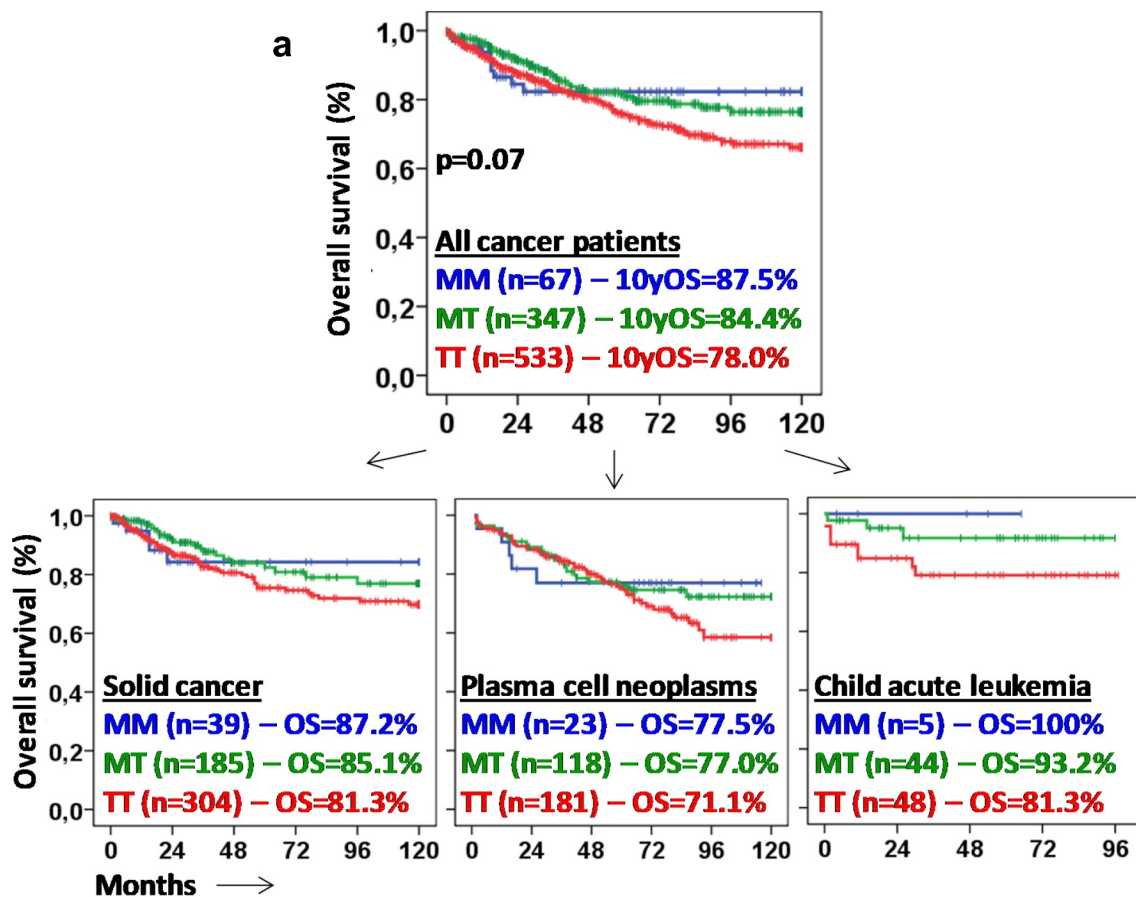

Cox regression for overall survival	HR	95% CI	P
Sex	0.916	0.637-1.318	0.638
Age	1.020	1.007-1.032	**0.002**
Type of cancer	1.023	0.953-1.098	0.526
HLA-B -21M/T dimorphism (TT, MT & MM)	1.422	1.015-1.992	**0.040**
C1/C2 HLA-ligands (C1C1, C1C2 & C2C2)	0.842	0.640-1.109	0.221
Bw4 HLA-ligands (Yes/No)	1.128	0.701-1.180	0.619

HR: hazard ratio; 95%CI: 95% confidence interval

Figure 3. Methionine −21 of the HLA-B leader peptide is associated with longer survival in patients with different types of cancer. (a) Kaplan–Meier and log-rank test for overall survival according to the genotype TT, MT or MM of the position −21 of the HLA-B leader peptide in all cancer patients and in patients with solid cancer (melanoma, bladderand ovarian cancer), plasma cell neoplasms and child acute leukemia. Ten-year overall survival rate (10yOS) is indicated for each group of patients. (b) Cox regression analysis of overall survival for sex, age, type of cancer, HLA-B −21 M/T dimorphism, and C1, C2, and Bw4 HLA-ligands.

Methionine −21 is associated with higher proliferation and lower expression of TIGIT after anti-CD3/CD28 stimulation of CD8⁺ T cells

To evaluate the role of HLA-E/-21 M-mediated interactions in the functionality of CD8⁺ T cells, polyclonal *in vitro* stimulation of purified CD8⁺ T cells was performed with anti-CD3/CD28. CD8⁺ T cells from healthy donors with the MM genotype (−21 M homozygous) have higher proliferative capacity ($p < .001$) than those with MT and MM genotypes (Figure 4a, left) and their cells accumulated in the third and fourth proliferation rounds ($p < .01$) as compared to MT and TT, whose cells accumulated in the first and second rounds (Figure 4a, right).

Additionally, CD8⁺ T cells from MM and MT donors showed lower levels of T-cell immunoglobulin and ITIM domain (TIGIT) expression after *in vitro* proliferation than TT donors. This was mainly due to TIGIT overexpression in TT donors during all proliferative cycles, whereas in donors bearing −21 M (MM and MT) the expression of TIGIT was maintained at basal levels (Figure 4b and supplementary Figure 2).

No differences in the expression level of CD8, CD25, CD27, CD38, CD226, NKG2A or TIM3 were observed overall or during the proliferative cycles in CD8⁺ T cells from the three genetic groups (Figure 4c). However, higher expression of NGK2D and lower of CD45RA was detected in CD8⁺ T cells overall and in the first and second proliferative cycles in MM patients ($p < .05$, Figure 4d).

TIGIT suppresses proliferation of CD8⁺ T cells after anti-CD3/CD28 stimulation

To further investigate the role of TIGIT expression on CD8⁺ T cells, the proliferative capacity of CD8⁺ T cells was evaluated in TIGIT-positive and TIGIT-negative cells after *in vitro* stimulation with anti-CD3/CD28. CD8⁺TIGIT⁻ T cells showed significantly higher ($p < .01$) proliferative capacity (86.5 ± 7.8%, 88.1 ± 9.0% and 96.8 ± 6.2%) than CD8⁺TIGIT⁺ T cells (63.1 ± 6.3%, 64.5 ± 5.4% and 70.0 ± 6.3%) in TT, MT and MM, respectively (Figure 5). The figure shows histograms with the proliferation profile of CD8⁺TIGIT⁻ and CD8⁺TIGIT⁺ T cells for a representative donor of the TT, MTand MM genotypes.

Discussion

NK and CD8⁺ T lymphocytes are cytolytic effectors involved in cancer immune surveillance.[44] Most functions of NK cells are analogous to those of CD8⁺ T cells, including production of pro-inflammatory and regulatory cytokines[45] and cytotoxicity against infected or tumor cells.[46] Although both cells are dependent on HLA recognition for the acquisition of functional competency,[6] T cells have evolved to offer more specialized and specific responses and NK cells have maintained versatility and plasticity.[30] In fact, NK cells and CD8⁺ T cells have long been considered to represent, respectively, the innate and adaptive arms of tumor and anti-viral immune responses.[47] However, the separation of these cells with regard to their contributions to adaptive immunity has to be reconsidered due to the discovery of long-lived NK cells that exhibit an immunological memory[48] capable of rapid recall responses[49] and due to their capacity to recognize specific HLA/peptide complexes.[15,16] Conversely, the results presented in this study show that CD8⁺ T lymphocytes are in turn sensitive to the licensing signals involved in the education of NK cells and, therefore, suggest that the gap between both lineages could be even narrower. Thus, M/T dimorphism at position −21 of the HLA-B leader peptide, which, when presented in HLA-E, interacts with NKG2A and intervenes in the education of NK cells,[30] is associated with variations in the expression of CD226 in CD56^bright NK cells, but more specifically in CD8⁺ T lymphocytes in patients with different types of cancer. However, unlike NK cells, CD8⁺ T cells do not appear to be sensitive to dimorphisms in HLA-A, -Band -C that determine C1, C2 and Bw4 ligands recognized by KIR receptors.[7,18,50] These results would be indicative that CD8⁺ T lymphocytes, in addition to their education in the thymus, are sensitive to specific signals involved in NK cell education. And what is more relevant, these interactions could be related to the survival of cancer patients and the immune surveillance capacity of their CD8⁺ T cells. In fact, −21 M HLA-B allotypes conditioned TCR-mediated response of CD8⁺ T lymphocytes, potentiating their proliferative capacity and restricting the stimulation-induced TIGIT over-expression, which is an inhibitory receptor that, according to our own and others' results, can restrain the expansion of CD8⁺ T cells and modulate negatively their antitumor response.[51,52] A diagram summarizing our results is shown in Figure 6.

Although these results do not allow for establishing causality between the survival of cancer patients and the −21 M/T HLA-B dimorphism, and despite the fact that the expression of CD226 is associated with but not required for NK cell education,[53] our hypothesis is supported by results describing that NK cell education through inhibitory-receptor/ligand interaction is associated with a higher expression of CD226, that contributes to stabilizing NK/tumor cell immunological synapse and tumor cell elimination by cytotoxicity.[18] This is further reinforced by results of our group describing that higher CD226/KIR expression ratios were associated with longer survival of patients with melanoma, ovarianand bladder carcinoma.[54] Indeed, licensing interactions not only up-regulate the expression of CD226 but also reduce that of inhibitory-KIR receptors, and consequently shift the CD226/iKIR receptor ratio on NK cell membranes toward the activating receptor.[54] However, transformed cells can down-modulate licensing-driven CD226/iKIR rearrangements as a specific mechanism to escape NK cell immune surveillance.[54,55] Predominance of CD226 over inhibitory receptors, particularly in the case of TIGIT, is of crucial importance because both receptors compete for the same ligands on the tumor cell (Nectin-2 or CD112 and poliovirus receptor or CD155[55]) and the equilibrium between positive and negative interactions might result in either cytolytic or tolerogenic responses. This becomes even more relevant in the antitumor response because, in general, transformed cells do not express conventional ligands (CD80, CD86, etc.) for the CD28 T-cell co-stimulatory molecule and in these circumstances the CD226
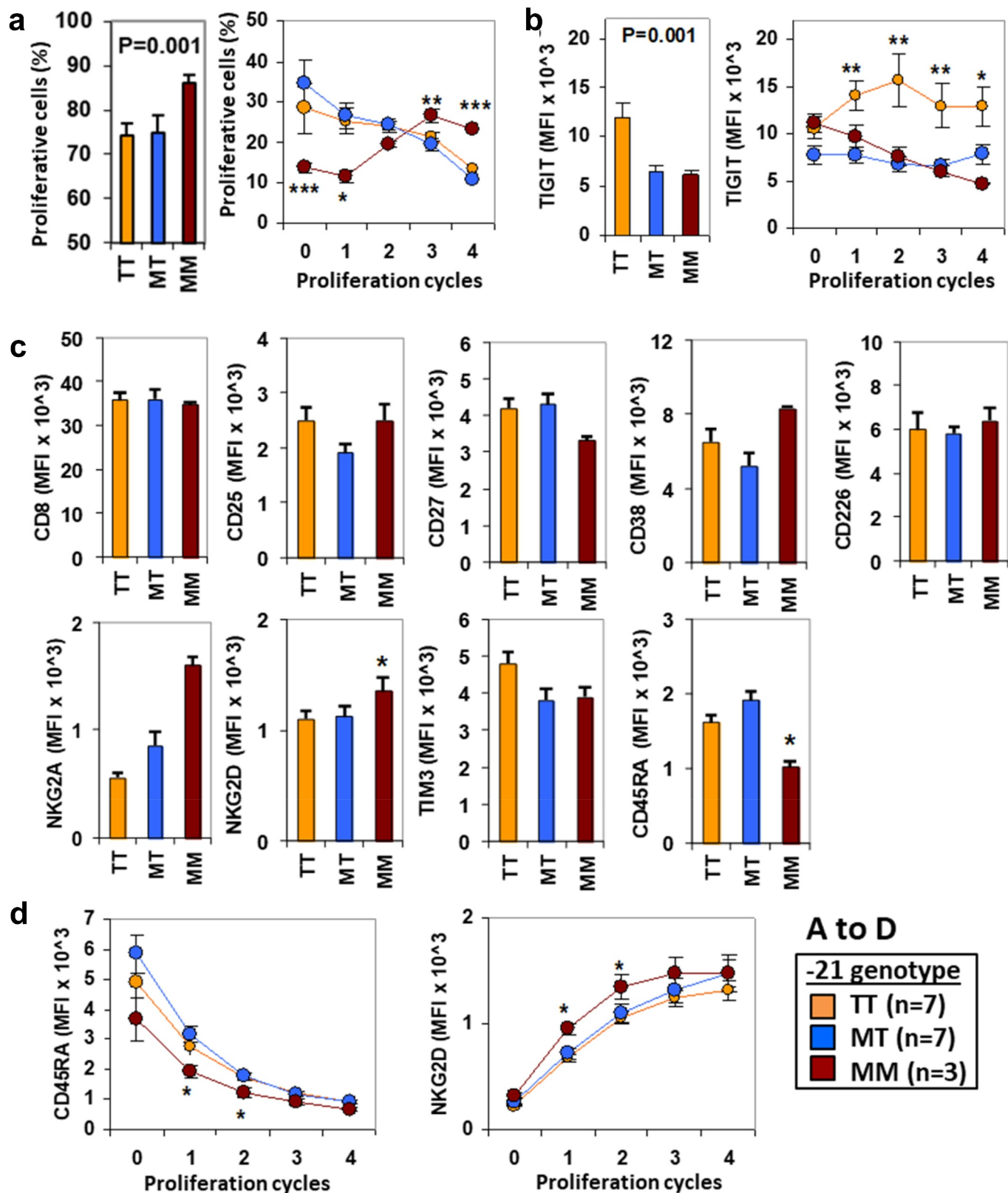
Figure 4. Methionine −21 is associated with higher proliferation and lower expression of TIGIT after anti-CD3/CD28 stimulation of CD8+ T cells. (a) Percentage of proliferative CD8+ T cells after anti-CD3/CD28 *in vitro* stimulation according to the −21 M/T genotype in total cells (left) or in cells within the proliferative cycles (right). Cells for each donor were assayed in triplicate and results are shown as mean±SEM. (b) Mean fluorescence intensity (MFI) of TIGIT on CD8+ T cells in similar conditions as in A. (c) Expression (MFI) of CD8, CD25, CD27, CD38, CD45RA, CD226, NKG2A, NGK2D TIM3, and CD45RA in total CD8+ T cells after *in vitro* stimulation with anti-CD3/CD28. (d) Expression (MFI) of CD45RA and NKG2D on CD8+ T cells within the proliferative cycles. * < 0.05, ** < 0.01and *** < 0.001 in the ANOVA and *post hoc* tests comparing each MM, MTor TT donors with the other two groups.
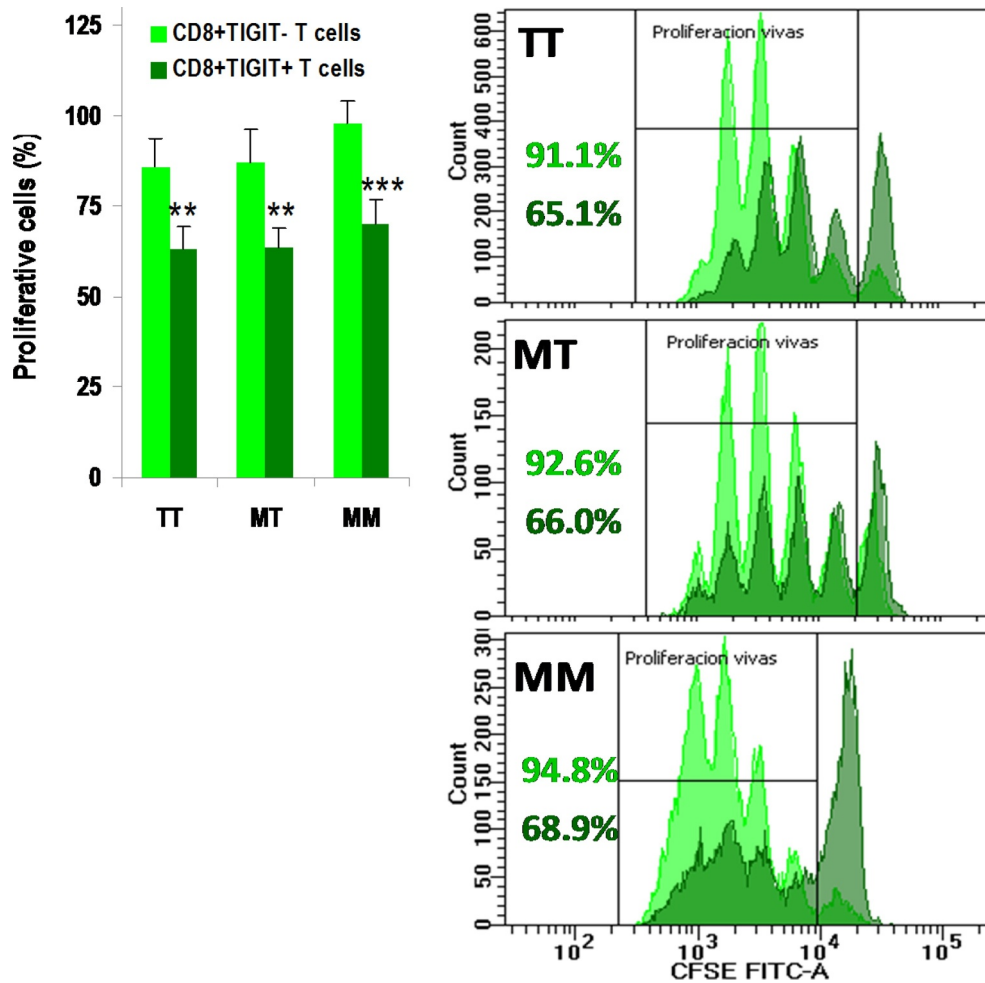
Figure 5. TIGIT suppresses proliferation of CD8$^+$ T cells after anti-CD3/CD28 stimulation. Percentage of proliferative cells within CD8$^+$TIGIT$^-$ (light green) and CD8$^+$TIGIT$^+$ (dark green) T cells after anti-CD3/CD28 *in vitro* stimulation, according to the −21 M/T genotype. Cells for each donor were assayed in triplicate and results are shown as mean±SEM. ** < 0.01 and *** < 0.001 in the Student's *t* test comparing CD8$^+$TIGIT$^-$ and CD8$^+$TIGIT$^+$ T cells. Representative proliferation histograms are shown for each genotype.
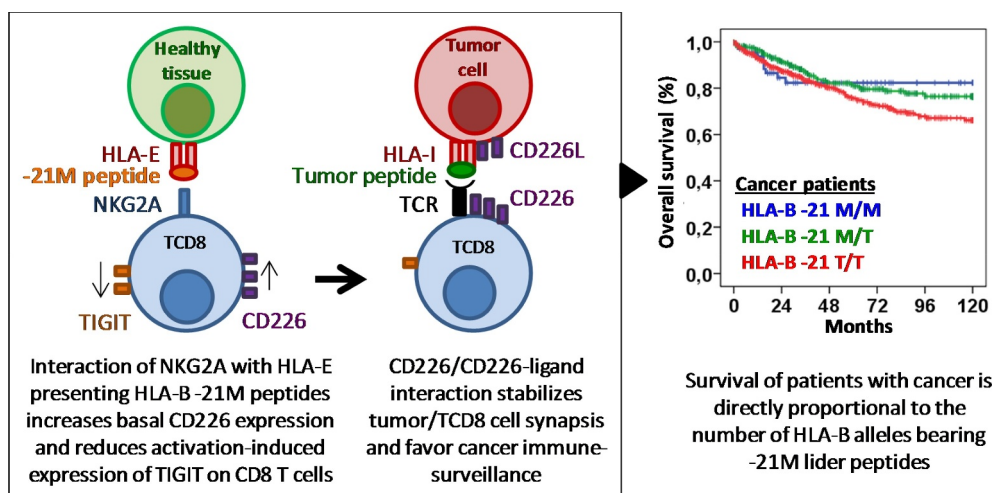
Figure 6.

co-stimulation could be decisive.[26,56,57] In fact, TIGIT blockade is one of the latest anti-checkpoint therapies included in clinical trials, trying to enhance the antitumor activity of T lymphocytes and NK cells.[58,59] Thus, the predictive value of HLA-B −21 M/T dimorphism, which is independent of patient´s age and cancer type, could contribute to identifying patients with a greater chance of responding to these new therapies. Nonetheless, this should be explored in new studies.

NK cells have two main pathways for education, one mediated by the interaction of inhibitory KIR with C1, C2 and Bw4 HLA-ligands, and the other mediated by NKG2A/HLA-E.[30] NKG2A/HLA-E pathway might prevail in −21 M individuals, around 45% of the global population, since this genotype is strongly associated with low frequency of C2 and Bw4 HLA-ligands,[30] and our own results are shown in Figure 2. But it might also be predominant in CD56^bright NK cells of all individuals since these cells do not express KIR receptors. The highly significant CD226 increase observed in the corresponding NK cell subsets in the presence of their specific C1, C2, Bw4 and −21 M ligands suggests that both education pathways are active in the NK cells of cancer patients. However, despite the fact that minimal expression of NKG2A on *ex vivo* or *in vitro* stimulated mature CD8+ T lymphocytes was observed (although sligtly higher in −21 M donors, see Figure 4c and supplementary Figure 2), the NKG2A/HLA-E pathway seems to be even more active in CD8+ T cells than in NK cells. This raises the question of how CD8+ T cells can sense the presence of −21 M/HLA-E complexes to induce the up-regulation of CD226. Although no simple answer can be given, it is possible that NKG2A could be interacting with HLA-E intracellularly, as reported for the Ly49 family of NK cell receptors in mice,[60] which are C-type lectin-like receptors, like the NKG2 human receptor family. Alternatively, it has been proven that CD8+ T cells recognize target cells in an HLA-E (in human[61]) or Qa-1b (in mice[62,63]) restricted, T-cell receptor-dependent manner. Nonetheless, we cannot rule out that CD226 expression on CD8+ T cells might be influenced by the interaction of NKG2A/HLA-E in other cells, such as NK cells, although our data indicate that the impact of NKG2A/HLA-E interaction on NK cells is weaker than in CD8+ T cells. Further research should be carried out to unravel molecular mechanims involved in this −21 M/HLA-E mediated CD226 up-regulation on CD8+ T cells.

Although educated in the thymus at their immature stages, mature T cells are still sensitive to mechanisms of peripheral tolerance.[63] It is therefore reasonable to think that they can also be sensitive to signals that educate and modulate the NK cell functioning. Unfortunately, the mechanisms by which the −21 M/T dimorphism of HLA-B influences CD226 expression and function of NK and CD8+ T cells are mostly unknown. Unlike NK cells, the main activating signal in T lymphocytes is MHC restricted and delivered by the TCR, but similar to NK cells, licensing signals could be modifying CD8+ T-cell metabolism,[19] reorganizing cytotoxic granules in their cytoplasm,[21] or shifting CD226/TIGIT ratio toward the activating receptor,[18,20] which could indeed favor more efficient responses in the T cells of individuals with −21 M alleles.

In conclusion, although these results need to be validated in new and larger series, they suggest that CD8+ T lymphocytes, like NK cells, are sensitive to the education induced by the M/T dimorphism at position −21 of the HLA-B leader peptide, which is reflected in the modulation of CD226 *in vivo* and in the proliferation and expression of TIGIT after *in vitro* stimulation, and overall it could be related to the immune surveillance capacity of CD8+ T lymphocytes and the survival of cancer patients. This might have relevant implications in the new checkpoint therapy blocking the inhibitory TIGIT receptor in diverse types of cancer.

Disclosure statement

The authors report no conflict of interest.

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