Original article

sHLA-G as a biomarker for colorectal cancer pathogenesis

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ABSTRACT

Objectives: Colorectal cancer (CRC) is a serious gastrointestinal disease. Cancer cells can survive in a microenvironment that includes distinct immune cells, various immune checkpoints including HLA-G, and different immune effectors such as interleukin-6 (IL-6), TNF-alpha (TNF-α), and interferon-gamma (IFN-γ). Thus, the objective of this study was to investigate HLA-G involvement in CRC as a prognostic factor in the Tunisian population, both in its soluble form (sHLA-G) and in its form linked to extracellular vesicles (HLA-G_{EV}). Additionally, we examined its association to the secretion of cytokines.
Methods: Fifty Tunisian patients, diagnosed with CRC, matched with ninety-eight healthy blood donors (HD) were enrolled in this study. Levels of sHLA-G, HLA-G_{EV}, IL-6, TNF-α, and IFN-γ were dosed in plasma samples using specific ELISA. For the functional assay, we assessed sHLA-G effects on the production of cytokines by stimulated T lymphocytes. We measured the concentration of IL-6, TNF-α, and IFN-γ produced by activated T lymphocytes pre-stimulated by plasma sHLA-G and HLA-G_{EV}.
Results: Our case-control analysis showed high concentration of both sHLA-G (8.8 [0–63] ng/ml vs. 2.1 [0–63] ng/ml, p < 0.0001) and HLA-G_{EV} (0.9 [0–17.8] ng/ml vs. 0.3 [0–31.0] ng/ml, p = 0.018) in CRC patients compared to HD. Concerning cytokines, IL-6 exclusively increased compared to HD (7.1[0.0–104.0] pg/ml vs. 3.6[0.0–58.22] pg/ml, p = 0.005). The evaluation of diagnostic performance using the receiver operating characteristic (ROC) curves and the area under the curve (AUC) showed sHLA-G (AUC = 0.828) as the more adequate biomarker in colorectal carcinoma prediction compared to HLA-G_{EV} (AUC = 0.615) and IL-6 (AUC = 0.664). We found that sHLA-G is able to decrease IL-6, TNF-α, and IFN-γ production by stimulated T lymphocytes in a concentration-independent manner. Additionally, HLA-G_{EV} appears to downregulate solely IL-6 release by stimulated T lymphocytes. Interestingly, HLA-G_{EV} was related to lymph node infiltration, which represents the most common metastatic route for colorectal cancer via the lymphatic system.
Conclusions: We outlined the importance of sHLA-G and HLA-G_{EV} as substantial immune biomarkers in CRC. Our findings suggest that HLA-G could be an effective diagnostic tool for colorectal cancer.

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1. Introduction

Colorectal cancer (CRC) is one of the most prevalent digestive cancers in the world. According to the latest Global Cancer Statistics report, almost 2 million new CRC cases and 935.000 deaths were registered in 2020 (Sung et al., 2021). Eventually, the rates of CRC-associated mortality are estimated to reach between 60 and 70% by 2035 (Araghi et al., 2019). There are several biological, genetic (e.g. mutations in anti-suppressor effectors), and molecular

(e.g. oxidative stress and inflammation through cytokine secretion) approaches implicated in CRC susceptibility and outcome (Barral et al., 2016, Kolligs, 2016, Begum et al., 2021).

Over the last decades, many reports have explored the relation between the immune system and cancer. Indeed, several mechanisms might empower extensively the tumor spread including the modulation of the CD8+ T lymphocytes (CTLs) and natural killer (NK) cells cytotoxicity, the impairment of B lymphocytes and dendritic cells (DCs) functions leading to an immune-tolerant tumor microenvironment (Gonzalez et al., 2018). Immunologists underlined an important cancer immunoediting process during which the immunity has dual opposite functions. In the early phases of tumor development, body defenses discard and obstruct as a general rule abnormal cell growth, whereas in late phases, they appear to be exceptionally responsible for tumor progression (Vesely et al., 2011). Actually, malignant cells have intriguingly adopted numerous strategies to evade immune surveillance by i) avoiding immune recognition through the alteration of major histocompatibility complex (MHC) expression, ii) production of immunosuppressive cytokines, and iii) expression of programmed death-ligand 1 (PD-L1) or CD80/86, which enables interaction with the cognate receptors on T and NK cells hampering immune function (Gonzalez et al., 2018, Teng et al., 2015, Chen et al., 2021). Nevertheless, many questions still need answers concerning the strategy of the immune system in either fighting or sustaining malignancies.

Based on their morphological characteristics, restricted polymorphisms, and limited cellular distribution, HLA-G is classified as a non-classical HLA class I molecule (Carosella et al., 2008). The examination of its structural diversity allows to categorize HLA-G within two major groups: membrane-bound (mHLA-G) and soluble molecules (sHLA-G) (Carosella et al., 2008). The final isoforms consist of proteins released into different biological fluids as free sHLA-G generated either by alternative splicing such as HLA-G5 (Carosella et al., 2008) or cleaved through proteolytic shedding by matrix metalloproteinases (MMPs) such as shed HLA-G1 (Park et al., 2004a). Additionally, Riteau and co-authors demonstrated that HLA-G can also be secreted via extracellular vesicles (EV; HLA-G$_{EV}$) in patients with melanoma (Riteau et al., 2003). Following their discovery, numerous efforts have been undertaken to better understand the accurate function of HLA-G. The interaction of HLA-G and its corresponding receptors, the immunoglobulin like transcript (ILT) receptor-2 (LILRB1/CD85j) and ILT4 (LILRB2/CD85d), and Killer cell immunoglobulin-like receptor 2DL4 (CD158d), triggers enormous effects modifying innate and adaptive immune responsiveness (Carosella et al., 2008, Morandi et al., 2007, Rouas-Freiss et al., 2003). For instance, HLA-G was found to restrain CTLs and NK cells cytotoxicity, to generate regulatory T lymphocytes (Tregs) and to down-regulate the alloproliferative response of CD4+ T lymphocytes (Carosella et al., 2008, LeMaoult et al., 2005, Chen et al., 2021). Initially, the role of HLA-G was thought to be limited to fetus-maternal interface and grafts tolerances (Rebmann et al., 2014). More recently, the contribution of sHLA-G was recognized in multiple respiratory (Cao et al., 2011), gynecological (Ben Yahia et al., 2018, Rebmann et al., 2003) and digestive (Cao et al., 2011, Lin et al., 2010) tumors. Although there is clear evidence indicating an aberrant up-regulation of sHLA-G expression in colorectal cancer (Cao et al., 2011, Zhu et al., 2011), some studies, on the contrary, have discarded any potential association between this molecule and CRC (Leelawat et al., 2004) or its clinicopathological characteristics (Zhu et al., 2011).

Recent experiments have identified EV as pivotal effectors in intercellular communication through harboring molecules (Rebmann et al., 2016). Furthermore, there are arising data claiming the implication of HLA-G$_{EV}$ in tumor progression (Rebmann et al., 2016). Grange and colleagues showed the capacity of HLA-G$_{EV}$ to hinder monocyte differentiation and proliferation to DCs, thereby disrupting the activation of T lymphocytes in renal cancer (Grange et al., 2015). Thus, it is suggested to perform future studies to discriminate between HLA-G as a free soluble molecule or as part of EV to understand the impact of these two soluble forms on malignant transformation and progression (Grange et al., 2015, Rebmann et al., 2016). Furthermore, some authors showed that these two forms of soluble HLA-G appear to promote adverse prognostic effects under pathological circumstances (Rebmann et al., 2016). The interaction between HLA-G and cytokines has progressively gained much attention. sHLA-G was considered to be a critical modulator ensuring the equilibrium between the production of T helper 1 (Th1) and T helper 2 (Th2) cytokines (Kanai et al., 2001, Kapasi et al., 2000, van der Meer et al., 2007), which was found to reciprocally regulate sHLA-G within pathophysiological events (Moreau et al., 1999, Sebti et al., 2007, Persson et al., 2020). Many reports have demonstrated that IL-6 and TNF-α might interfere in cancer initiation and migration along with their essential role in angiogenesis and recurrences (Mager et al., 2016). Moreover, some studies have underlined that IFN-γ seems to limit the multiplication of abnormal colonic cells and thereby impair neoplasms progression and dissemination (Castro et al., 2018, Mager et al., 2016).

Based on this knowledge, our study aims to evaluate the implication of secreted forms of HLA-G including soluble HLA-G (sHLA-G) as well as plasmatic HLA-G linked to EV (HLA-G$_{EV}$), in CRC development and progression. In addition, we explored the relationship of these two soluble HLA-G forms with pro-inflammatory cytokines (IL-6, TNF-α and IFN-γ).

2. Materials and Methods

2.1. Population data and blood collection

Fifty Tunisian patients diagnosed with CRC (34 males and 16 females; mean age ± SEM (Standard Error of the Mean): 60.38 ± 2.085 years; range: 29–85 years) at the Institute of Salah Azaïz of Tunis (ISA, Tunisia) were enrolled in this study. The control group consisted of age- (unpaired *t*-test P = 0.188) and gender- (unpaired *t*-test P = 0.941) matched ninety-eight healthy donors (HD; 67 males and 31 females; mean age ± SEM: 57.16 ± 1.381 years; range: 27–89 years). After centrifugation of blood samples at 3500 rpm (round per minute) for 10 min (minutes) at 4 °C, plasma supernatants of the study population were stored at −80 °C until further analysis. None of the patients have undergone surgical intervention or have been the subject of neo-adjuvant chemotherapy and/or radiotherapy treatments during blood collection.

On the basis of the criteria recommended by the American Joint Committee on Cancer (AJCC, eighth edition) and the World Health Organization (WHO, fifth edition), we documented clinicopathological parameters for each patient including tumor-node-metastasis (TNM) classification, location, tumor size, histological type, and degree of differentiation. We documented also gender, age, familial antecedents, and lifestyle habits (alcohol, smoking) for each patient.

All patients and volunteers have given their written consent to participate in the present study. This work was approved by the ethics committee of ISA (Ref N°: ISA/2016/3).

2.2. Quantification of sHLA-G and HLA-G$_{EV}$

Quantification of plasmatic sHLA-G and HLA-G$_{EV}$ concentrations was performed according to the Essen Workshop protocol for soluble HLA-G measurement (Rebmann et al., 2005). The total amount

of sHLA-G and HLA-G$_{EV}$ after EV enrichment was quantified in plasma samples or EV preparation in a 1:1 dilution by sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA) technique. Purified HLA-G1 was used as standard reagent (Celik et al., 2018). The monoclonal antibody (mAb) MEM-G9 (Exbio, Praha, Czech Republic) was used as capture antibody to target both sHLA-G and HLA-G$_{EV}$. The detection antibody was a polyclonal rabbit anti-human beta-2-microglobulin (Dako, Glostrup, Denmark). All ELISA assays were assessed in duplicates. Absorbance values were measured via BioTek™microplate reader at 450 nm and then analyzed with Gen5™data collection and analysis software (version 1.11). The intra- and inter-assay variation of the ELISA was 2.5% and 16.9%, respectively.

2.3. Isolation of extracellular vesicles (EV)

EV were extracted from plasma samples using the ExoQuick™ precipitation solution (SBI Systems Bioscience, Palo Alto, CA, USA) as previously reported (König et al., 2016; Schwich et al., 2019). At the outset, we weighted 63 μg of ExoQuick™ solution in a sterile 15 ml conical Falcon tube. A total volume of 250 μl of plasma samples was then added to the ExoQuick™ solution. After being mixed, tubes were incubated overnight (at least 12 h) at 4 °C for EV precipitation and then centrifuged at $1500 \times g$ for 30 min. Supernatant was discarded and the remaining pellet of enriched EV was resuspended in 25 μl Aqua-dest and adjusted with 0.9% NaCl to a final volume of 250 μl.

2.4. Isolation of plasma derived sHLA-G and HLA-G$_{EV}$

To mirror as close as possible the in-vivo situation of tumor patients, we enriched sHLA-G molecules and HLA-G$_{EV}$ from plasma samples of patients having low (sHLA-G < 10 ng/ml, HLA-G$_{EV}$ < 5.0 ng/ml), middle (sHLA-G:10–20 ng/ml, HLA-G$_{EV}$: 5–10 ng/ml), and high HLA-G levels (sHLA-G > 20 ng/ml, HLA-G$_{EV}$ > 10 ng/ml). Plasmatic sHLA-G and HLA-G$_{EV}$ molecules from enriched EV fractions were isolated according to a standard protocol with minor modifications (Verloes et al., 2011, Veit et al., 2015). The anti-human HLA-G mAb G233 (Exbio) was coupled to Dynabeads® MyOne™ Carboxylic Acid (Thermofisher, Germany) according to the instruction of the manufacturer. Thereafter, beads (20 000) were incubated either with 110 μl EDTA plasma samples (N = 13) or with EV preparations (N = 16) overnight at room temperature. As a negative control, G233 coupled beads were incubated with complete medium (CM: RPMI 1640, Invitrogen, Karlsruhe, Germany) containing 1% penicillin/streptomycin, 200 μg/mL L-glutamine and 10% fetal calf serum. After two washing steps with PBS buffer containing Penicillin/Streptomycin, HLA-G loaded beads were adjusted to a final volume of 20 μl with CM. The sHLA-G unloaded microspheres (negative control) themselves did not influence functional analysis.

2.5. Priming of T lymphocytes with sHLA-G and CD28/CD3 T lymphocytes stimulation

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from heparinized density gradient centrifugation using Lymphoprep™ (Alere Technologies AS, Oslo, Norway). To prime PBMCs with HLA-G (sHLA-G/HLA-G$_{EV}$), we added 20 μl sHLA-G/HLA-G$_{EV}$ loaded or unloaded beads to the freshly isolated 50.000 PBMCs and cultured the suspension in a volume of 200 μl CM in 96 wells round-bottom plates. After 18 h of culture, sHLA-G/HLA-G$_{EV}$ primed cells were stimulated with beads coated with CD28/CD3 (Thermo Fisher Scientific, Lithuania) in a beads:cell ratio of 1:3 for 48 h (Schwich et al., 2020). Thereafter supernatants were

immediately collected and stored at −25 °C until further analysis. Each sample was tested in duplicates.

2.6. Quantification of cytokines in plasma and cell supernatants

We determined plasmatic levels of IL-6 (Invitrogen™ Human IL-6 ELISA Ready-SET-Go!™, ThermoFisher Scientific, San Diego, CA, USA), TNF-α (Invitrogen™ Human TNF-α ELISA Ready-SET-Go!™, ThermoFisher Scientific, San Diego, CA, USA) and IFN-γ (Invitrogen™ Human IFN gamma Uncoated ELISA Kit, Thermo-Fisher Scientific, Vienna, Austria) in CRC patients and HD, with commercial sandwich ELISA kits according to producer's guidelines. Optical density was spectrophotometrically estimated at wavelength of 450 nm, and concentrations were determined using a standard curve provided for each kit.

Diluted supernatants upon activation of primed T lymphocytes with CD28/CD3 Dynabeads, were additionally tested for their content of IL-6, TNF-α, and IFN-γ cytokines. All cytokines were measured in duplicates.

2.7. Data analysis and statistics

Statistical analyses were performed using Graphpad Prism (version 8.0.1) and IBM SPSS Statistics (version 21.0). Levels of sHLA-G, HLA-G$_{EV}$, and cytokines are given as median and range. After testing for Gaussian distribution differences of HLA-G and cytokines expressions between patients and HD and their impacts on colorectal cancer clinicopathological features were assessed by the Mann Whitney test, Chi-square (X^2) test or Spearman's correlation. A p-value below 0.05 was considered as statistically significant. To evaluate whether the analyzed parameters are meaningful markers, to distinguish between patients and HD, the receiver operating characteristic (ROC) and the area under the curve (AUC) were examined by the NCSS statistical software (version 20.0.3).

3. Results

3.1. Baseline characteristics of CRC population

Clinical and demographic parameters of patients included in this research are shown in Table 1. Overall, the average age of patients at disease diagnosis was 60 years (Range: 29–85 years). Histologically, adenocarcinoma (94%) was the main identified subtype. 60% of patients were at T3 stage. The invasion of tumors into regional lymph nodes (N +) has occurred in 64% of cases. The majority of patients (n = 31) had no signs of extra-colorectal metastases. Among those diagnosed with distant dissemination, cancerous cells have predominantly spread towards hepatocellular tissues. According to TNM staging classification, we distinguished hence four stages distributed as following: stage I (6%), stage II (16%), stage III (40%), and stage IV (38%). More than two-thirds of patients had tumors strictly larger than 4 cm. We noted three grades of malignant cell differentiation: well- (66%), moderately (24%), and poorly (4%) differentiated.

3.2. Increased levels of sHLA-G, HLA-G$_{EV,}$ and IL-6 in CRC patients

To investigate whether sHLA-G and HLA-G$_{EV}$ molecules are involved in CRC disease, we evaluated the concentration of these parameters in plasma samples from patients with CRC and from HD (Table 2). Overall, significant high levels of sHLA-G (Median [Range]: 8.8 [0–63] ng/ml vs. 2.1 [0–63] ng/ml; p < 0.0001) and HLA-G$_{EV}$ (0.9 [0–17.8] ng/ml vs. 0.3 [0–31.0] ng/ml; p = 0.018) were found in CRC patients compared with HD (Fig. 1a, b). Interestingly, HLA-G was undetectable in 43.9% of the EV fractions (43/98)

Table 1
Baseline characteristics of patients with colorectal cancer.

Variables	Patients (N = 50)
Age at disease diagnosis (years)	
Median	62.5
Range	29-85
Gender	
Males	34 (68%)
Females	16 (32%)
Tumor location	
Rectum	34 (68%)
Colon	16 (32%)
Histological type	
Adenocarcinoma	47 (94%)
Others	3 (6%)
Differentiation degree status	
Well + moderate	45 (90%)
Poor	2 (4%)
UK	3 (6%)
Tumor depth invasion (T) status 1	
T1+T2	9 (18%)
T3+T4	40 (80%)
UK	1 (2%)
Tumor depth invasion (T) status 2	
T1+T2+T3	39 (78%)
T4	10 (20%)
UK	1 (2%)
Regional Lymph Nodes (N)status	
N0	15 (30%)
N+	32 (64%)
UK	3 (6%)
Metastasis (M)	
M0	31 (62%)
M1	19 (38%)
TNM (1)	
I+II	11 (22%)
III+IV	39 (78%)
TNM (2)	
I+II+III	31 (62%)
IV	19 (38%)
Tumor size (cm)	
>4	38 (76%)
≤4	11 (22%)
UK	1 (2%)

Data are illustrated in numbers (n) and percentages (%) median, and ranges. TNM: tumor-nodes-metastasis. UK: unknown.

derived from HD, whereas only 30% (15/50) of the EV fractions of CRC patients contained no HLA-G.

The IL-6 median level was nearly twofold enhanced in CRC patients compared to HD (Respectively: 7.1 [0–104] pg/ml vs. 3.6 [0–58.22] pg/ml, p = 0.005; Fig. 2a). However, no differences in

IFN-γ and TNF-α levels were reported for CRC patients compared to HD (Fig. 2b, c).

3.3. Correlation of sHLA-G, HLA-G$_{EV}$ and cytokine levels

sHLA-G and HLA-G$_{EV}$ levels were positively correlated in CRC patients (r = 0.689, p < 0.0001). sHLA-G was inversely correlated with TNF-α levels (r = -0.446, p = 0.001). However, HLA-G$_{EV}$ was not correlated to TNF-α (r = −0.223, p = 0.123). Furthermore, IFN-γ and IL-6 levels were not correlated neither with sHLA-G nor HLA-G$_{EV}$ levels (p > 0.05).
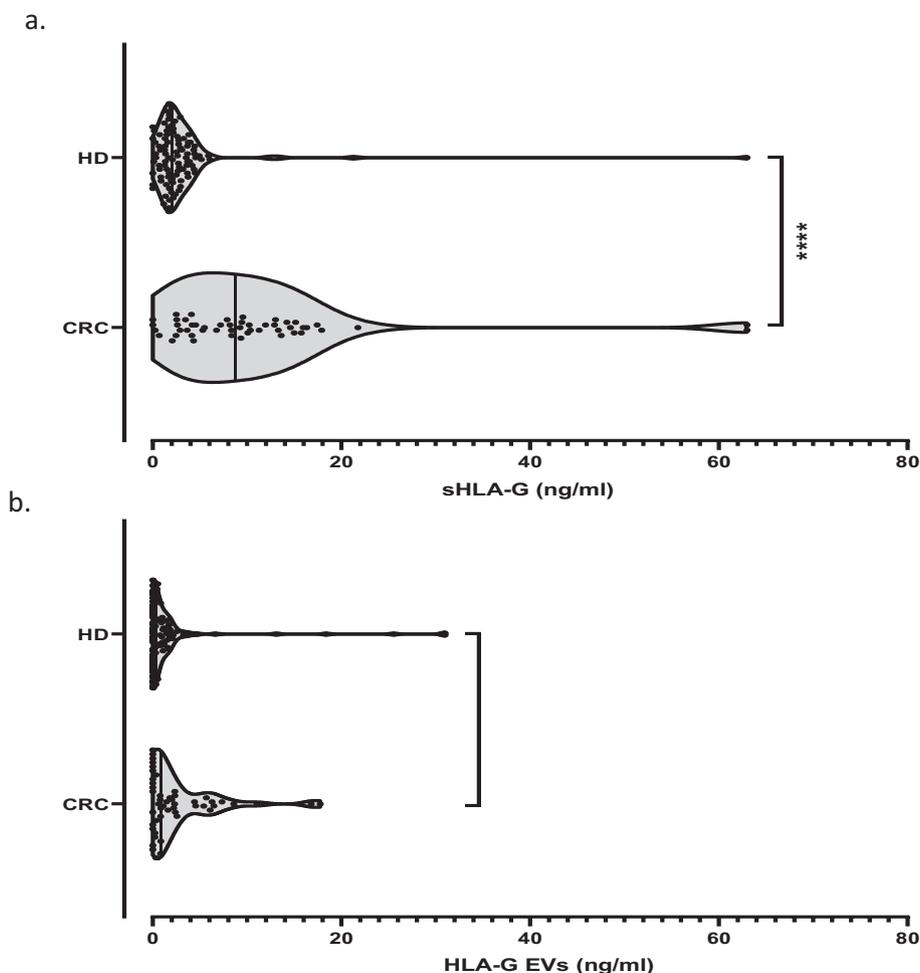
3.4. Correlation of HLA-G with demographic and clinicopathological CRC patients' features

To assess whether the differences in HLA-G production are related to demographic and CRC clinicopathological features, we stratified patients into subgroups according to age, gender, tumor size, location, TNM staging, histological type, and degree of differentiation (Table 3). In spite of insignificance, enhanced levels of sHLA-G and HLA-G$_{EV}$ were observed among T4 stage CRC patients. Even as no significant median differences was retrieved, levels of both, sHLA-G and HLA-G$_{EV}$ were enhanced in patients aged above 62 years.

To further study the effect of aging on HLA-G and cytokines levels, we divided our population into three groups: young (27–39 years), middle-aged (40–64 years) and elderly CRC patients (≥65 years) (Nematolahi and Ayatollahi, 2017) (Table 5). In comparison with HD, young, middle-aged, and elderly CRC patients significantly expressed high levels of sHLA-G (p < 0.05). Additionally, elderly CRC patients showed increased levels of HLA-G$_{EV}$ and decreased TNF-α levels compared to HD (Respectively: p = 0.012; p = 0.009, Table 5). Middle-aged patients showed higher IL-6 levels than HD (p = 0.036). No significant differences for IFN-γ were found between CRC patients and HD (Table 5).

While both female and male patients have expressed almost similar levels of HLA-G$_{EV}$, we noticed increased levels of sHLA-G in the men group without reaching significance (9.5 [4.5–13.97] ng/ml vs. 6.2 [2.48–13.23] ng/ml, p = 0.118, Table 3). Levels of sHLA-G and of HLA-G$_{EV}$ were not correlated with any clinicopathological parameters (p values > 0.05, Mann Whitney U test, Table 3).

Using the median values of sHLA–G or HLA-G$_{EV}$ as cut-off for the stratification of patients into high and low producers, respectively, we demonstrated that HLA-G$_{EV}$ and sHLA-G/HLA-G$_{EV}$ ratios were significantly related to lymph nodes infiltration (Respectively, Chi square: p = 0.030, p = 0.029; Table 4).

Table 2
Association of sHLA-G, HLA-G$_{EV}$, IL-6, TNF-α and IFN-γ with CRC risk.

Parameters	N	Median(range)	IQR (25%–75%)	P-value (Mann-Whitney)
sHLA-G (ng/ml)				
Patients	50	8.8 (0–63)	3.99–13.8	**<0.0001**
Controls	98	2.1 (0–63)	1.4–3.6	
HLA-G$_{EV}$(ng/ml)				
Patients	50	0.9 (0–17.8)	0.0–3.0	**0.018**
Controls	98	0.3 (0–31)	0.0–1.5	
IL-6 (pg/ml)				
Patients	49	7.1 (0–104.0)	3.0–13.4	**0.005**
Controls	50	3.6 (0–58.22)	1.1–6.6	
TNF-α (pg/ml)				
Patients	49	1.8 (0–157.5)	0–19.2	0.359
Controls	95	7.8 (0–218.4)	0–24.2	
IFN-γ (pg/ml)				
Patients	50	0 (0–1.2)	–	0.264
Controls	98	0 (0–357.9)	–	

sHLA-G: soluble HLA-G; EV: extracellular vesicles; IL: interleukin; TNF-α: tumor necrosis factor-alpha; IFN-γ: Interferon-gamma; N: number; IQR: interquartile range 25–75%; all P values are estimated using the Mann-Whitney test.

Fig. 1. Comparison of sHLA-G and HLA-G$_{EV}$ concentrations between CRC patients and healthy donors (HD). Statistical differences of sHLA-G (a) and HLA-G$_{EV}$ (b) levels between patients and HD were evaluated by the Mann–Whitney *U* test. Concentrations are expressed in ng/ml. *, ****: P values ≤ 0.05 and ≤ 0.0001 respectively.

3.5. Clinical implication of HLA-G and cytokines in CRC diagnosis

The efficiency of HLA-G and cytokines as potential markers in CRC diagnosis was defined by the ROC curve analysis (Fig. 3). sHLA-G showed a good performance in the distinction between CRC patients and HD (AUC = 0.828, 95% CI: 0.728–0.894, p < 0.0001). In fact, sHLA-G had 76% of sensitivity and 84.7% of specificity at the cut-off concentration of 4.10 ng/ml. Similarly, HLA-G$_{EV}$ indicated a good performance in the distinction between CRC patients and HD (AUC = 0.615, 95% CI: 0.510–0.702, p = 0.009).

Regarding cytokines, IL-6 displayed an AUC of 0.664 (95% CI: 0.541–0.759, p = 0.001). For a cut-off value of 6.46 pg/ml, IL-6 shows a sensitivity of 55% and a specificity of 76% for CRC detection. TNF-α and IFN-γ were no meaningful parameters in CRC discrimination.

3.6. sHLA-G reduces cytokines secreted by T lymphocytes in a concentration-independent manner

The potential involvement of sHLA-G in the proliferation of T lymphocytes and their cytokines production was then investigated. We used CRC plasma samples expressing different quantities of sHLA-G and HLA-G$_{EV}$. Once stimulated with CD28/CD3 activator beads, we measured the levels of secreted IL-6, TNF-α, and IFN-γ cytokines by HLA-G-primed T lymphocytes using the ELISA sandwich technique. Although, we used different concentrations of

sHLA-G and HLA-G$_{EV}$ to estimate their impacts on the ability of T lymphocytes to secrete cytokines, we found that sHLA-G decreased the expression of all measured pro-inflammatory cytokines in a dose-independent manner (p values < 0.05; Fig. 4a,b,c). However, HLA-G$_{EV}$ significantly reduced only the IL-6 production in an independent-dose manner (p = 0.0018; Fig. 4d), whereas no relevant effect of HLA-G$_{EV}$ priming was observed for TNF-α and IFN-γ (p > 0.05; Fig. 4e,f).

4. Discussion

Most tumors have developed fundamental strategies to escape immune surveillance. Indeed, the transition of anormal colonic mucosa into an oncogenic phenotype entail constitutive activation of NF-κB pathway, abolishment of anti-apoptotic proteins (Bcl-2) together with redox imbalance (Begum et al., 2021, Uddin et al., 2021). In addition, HLA-G and its soluble forms are considered to be potent biomarkers in several pathologies including colorectal cancer (Heidari et al., 2017, Li et al., 2017, Pan et al., 2016). To date, these molecules were the objects of multiple studies revealing that their expression could be reliable to discriminate between colorectal malignant transformations and benign or normal contexts (Zhu et al., 2011). In the present study, we demonstrate that CRC samples exhibited higher levels of sHLA-G than HD (p < 0.001) in accordance with previous publications (Farjadian et al., 2018, Lázaro-Sánchez et al., 2020, Li et al., 2017). Another article has announced
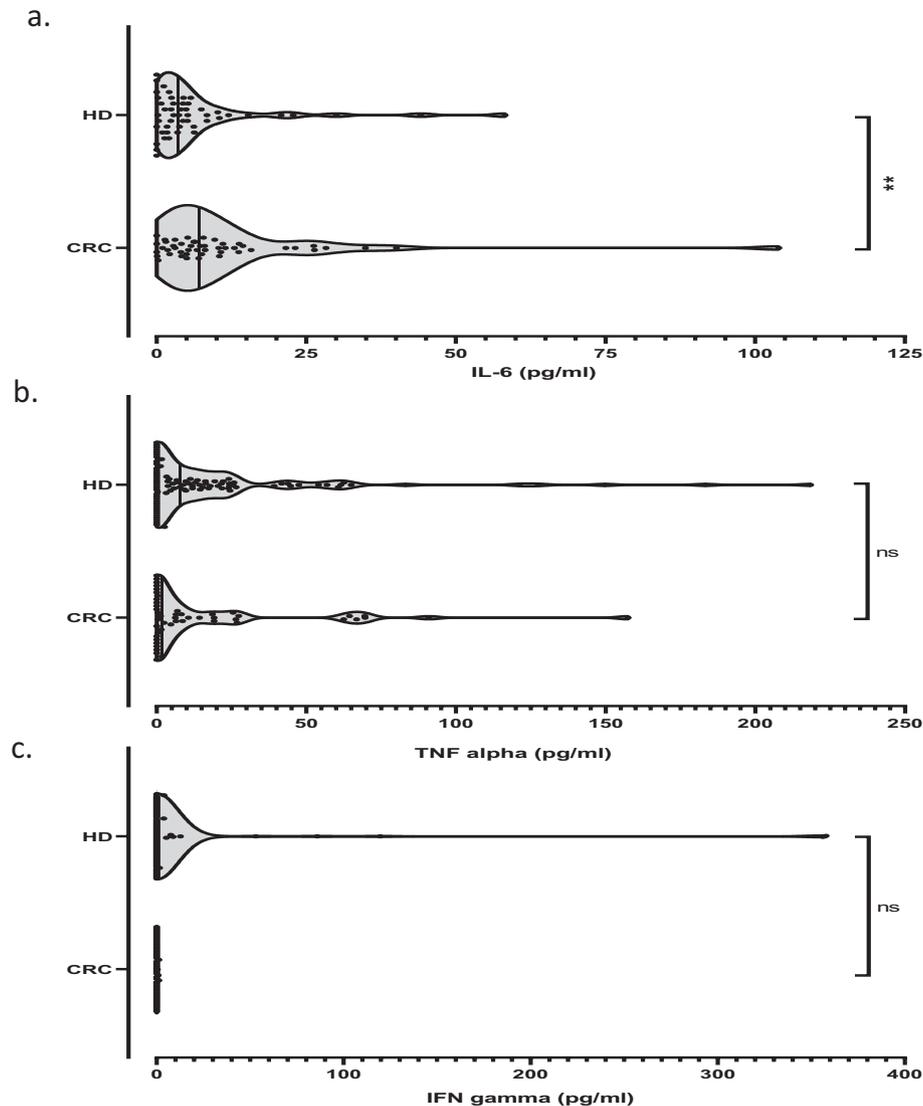
Fig. 2. Comparison of IL-6, TNF alpha and IFN gamma concentrations between CRC patients and healthy donors (HD). The distribution of IL-6 (a), TNF alpha (b) and IFN gamma (c) levels between patients and HD were estimated by the Mann–Whitney U test. Cytokine amounts are expressed in pg/ml. **: P value $\leq$ 0.01. Ns: non significant.

that sHLA-G was highly expressed among CRC cases compared to normal subjects but only with borderline significance (p = 0.058) (Abu Hassan et al., 2019). Lázaro Sánchez and co-workers provided evidence of significant production of sHLA-G in stages III and IV colorectal cancer (Lázaro-Sánchez et al., 2020), whereas others claimed greater secretion of sHLA-G predominantly in earliest stages to boost the multiplication and survival of malignant cells (Farjadian et al., 2018). Our results revealed no correlation of sHLA-G levels with CRC stage, location, grade, lymph nodes infiltration, and metastasis likewise to the study of Zhu et al. (2011). Instead, we noticed a slight increased secretion of sHLA-G in T4 stage CRC without significance as previously observed among advanced primary tumors depth (Li et al., 2017). Other authors found that sHLA-G was correlated to some clinicopathological features proposing it as a prominent prognostic factor during CRC (Kirana et al., 2017, Lázaro-Sánchez et al., 2020, Li et al., 2017). The ethnicity and the reduced number of our population might explain this discrepancy with previous results.

Extracellular vesicles are secreted via normal and transformed cells (Rebmann et al, 2016). Following this finding, emerging

details have pointed out EV as essential factors to monitor mutual exchanges between cells and their environment during biological processes and pathogenesis (Rebmann et al., 2016). Subsequently, EV became promising elements to control and treat diseases. The constitution of EV depends on their secretory cells, contexts of release, and functions (Rebmann et al., 2016). In this concept, a large spectrum of molecules such as HLA-G (Riteau et al., 2003), cytokines, micro-RNAs, and lipids (Rebmann et al., 2016) were found enclosed in EV. Recently, HLA-G$_{EV}$ were significantly associated with colorectal carcinogenesis, meanwhile correlated with tumor status, lymph node metastases as well as with TNM staging (Zhao et al., 2019). Here, we unveil that HLA-G$_{EV}$ was overproduced among CRC patients, yet we failed to establish any correlation between this mediator and the majority of clinicopathological features of the disease. However, enhanced amounts of HLA-G$_{EV}$ were reported among CRC patients with T4 primary tumors. Moreover, we found that 83% of patients with enhanced levels of HLA-G$_{EV}$ were diagnosed with positive lymph nodes (p = 0.030). The sHLA-G/HLA-G$_{EV}$ ratio was significantly related to lymph node infiltration (p = 0.029), which represents the most common meta-

Table 3

Association of clinicopathological data with the expression of sHLA-G and HLA-G $_{EV}$ in patients with colorectal cancer.

Variables	N	sHLA-G (ng/ml) median (IQR)	P$_1$-value	N	HLA-G $_{EV}$ (ng/ml) median (IQR)	P$_2$-value
Age at disease diagnosis (median, years)						
>62	25	9.550 (6.840–14.11)	0.111	25	1.810 (0.120–4.975)	0.171
≤62	25	5.310 (2.765–13.10)		25	0.780 (0–2.260)	
Gender						
Males	34	9.500 (4.505–13.97)	0.118	34	0.850 (0.180–4.453)	0.43
Females	16	6.175 (2.480–13.23)		16	0.615 (0–2.513)	
Tumor location						
Rectum	34	8.715 (3.608–13.77)	0.546	34	0.835 (0–2.363)	0.48
Colon	16	8.910 (4.115–15.14)		16	0.990 (0.240–5.618)	
Histological type						
Adenocarcinoma	47	9.240 (4.160–14.23)	0.264	47	0.840 (0.-4.420)	0.641
Others	3	2.620 (2.460–11.29)		3	2.310 (0.360–2.350)	
Differentiation degree status						
Well + moderate	45	8.370 (4.130–13.93)	0.666	45	0.840 (0–4.485)	0.647
Poor	2	7.100 (2.910–11.29)		2	1.605 (0.860–2.350)	
Tumor invasion status (T)						
T1+T2	9	8.340 (1.570–11.55)	0.247	9	0.330 (0–3.430)	0.357
T3+T4	40	9.345 (4.165–14.91)		40	0.950 (0–3.395)	
T1+T2+T3	39	8.370 (4.16–13.62)				
T4	10	11.63 (2.818–16.17)	0.578	39	0.840 (0–2.400)	0.336
				10	1.605 (0.285–7.030)	
Regional Lymph Nodes status (N)						
N0	15	7.890 (4.180–13.01)	0.941	15	0.780 (0–2.340)	0.209
N+	32	9.300 (3.523–14.08)		32	1.535 (0–5.188)	
Metastasis (M)						
M0	31	9.360 (3.650–13.62)	0.886	31	0.800 (0–2.350)	0.412
M+	19	8.190 (4.100–14.60)		19	0.870 (0–5.690)	
TNM Staging						
I+II	11	10.08 (4.340–15.16)	0.484	11	0.780 (0–2.340)	0.427
III+IV	39	8.370 (3.480–13.62)		39	1.120 (0–4.550)	
I+II+III	31	9.360 (3.650–13.62)	0.886	31	0.800 (0–2.350)	0.412
IV	19	8.190 (4.100–14.60)		19	0.870 (0–5.690)	
Tumor size (cm)						
>4	38	9.405 (4.145–14.43)	0.322	38	0.990 (0–4.453)	0.919
≤4	11	5.310 (2.620–10.35)		11	0.840 (0–0.2400)	

P values calculated using the Mann-Whitney test, P1: P-value of sHLA-G, P2: P-value of HLA-G$_{EV}$; sHLA-G: soluble HLA-G; EV: extracellular vesicles; n: number; IQR: interquartile range 25–75%; Cm: centimeters.

static route of colorectal cancer via the lymphatic system (Zhang et al., 2020). Therefore, these results suggest that HLA-G$_{EV}$ might exert an important role in tumor progression, although we did not find any other significant association with clinicopathological factors of tumor progression.

In the current paper, we concluded that both sHLA-G and HLA-G$_{EV}$ might provide a favorable microenvironment for CRC development but only HLA-G$_{EV}$ might have a significant effect on disease progression. This could be due to the HLA-G form that differently modulates immune functions as previously suggested (König et al., 2016; Rebmann et al., 2016).

IL-6 is a multifunctional pro-inflammatory cytokine synthesized by lymphocytes, endothelial cells, and macrophages (Wang and Sun, 2014). Recent studies have unveiled the implication of this cytokine in solid cancers especially in colorectal malignancy (Wang and Sun, 2014). Indeed, IL-6 triggers the Janus kinase signal transducer and activator of transcription 3 (JAK/STAT3) pathway which orchestrates an accordingly pro-oncogenic machinery to maintain tumor cell viability and proliferation (Wang and Sun, 2014). In our cohort, we presumed that the observed overexpression of IL-6 might be related to CRC (p = 0.005) as previously reported (Kaminska et al., 2005, Kantola et al., 2012, Kim et al., 2019, Shiga et al., 2016).

Over time, a growing debate has addressed the role of age in cancer leading to the assumption that CRC constitutes a common issue for both young and old individuals. At the outset, closer insights into CRC causalities showed that aging triggers the accumulation of aberrations in cell activities and thus CRC has to be considered as a typical disease for elders (Nangia-Makker et al., 2015). Despite and except for hereditary genetic disorders, lifestyle habits and dietary were found to further arise the frequencies of sporadic CRC in young people (Sung et al., 2021). Concerning our CRC population, no correlation between colorectal tumorigenesis and age was found as hitherto announced (Kim et al., 2019, Li et al., 2017). Conversely, age-stratified analyses demonstrated important changes in biomarkers expression between CRC patients and HD. Indeed, we registered the greatest quantities of sHLA-G in youngest, middle-aged, and eldest CRC groups. Significant enhanced amounts of HLA-G$_{EV}$ were measured only for elderly patients.

Based on ROC analysis, both, sHLA-G and HLA-G$_{EV}$ might constitute accurate diagnostic tools for colorectal cancer assessment in accordance with aforementioned studies (Cao et al., 2011, Zhu et al., 2011; Zhao et al., 2019). In our study, sHLA-G (AUC = 0.828) was found to dispose more relevant ability in the differentiation between CRC patients and HD than HLA-G$_{EV}$ (AUC = 0.615). Even though CEA (carcino embryonic antigen) and Ca19-9 (Carbohydrate antigen 19–9) are available blood markers for the detection of CRC (Cao et al., 2011, Groblewska et al., 2008), it has shown that sHLA-G is a more reliable indicator than CEA to detect CRC (Cao et al., 2011). Although, there was noticeable deviation of IL-6 AUC scores ranging from 0.561 to 0.896 (Kantola et al., 2012, Pengjun et al., 2013, Bünger et al., 2011, Groblewska et al., 2008), the expression of IL-6 was reported to be predictive for CRC. In a meta-analysis on IL-6 performance status in CRC (Xu et al., 2016), authors declared an AUC of 0.79 (95% CI: 0.75–0.82), leading to propose this molecule as a moderate biomarker for disease identification. Likewise, our statistics revealed an

Table 4
Distribution of clinical parameters according to sHLA-G, HLA-G$_{EV}$, and sHLA-G/HLA-G$_{EV}$ ratio among patients with colorectal cancer.

Parameters	sHLA-G (ng/ml)			HLA-G$_{EV}$ (ng/ml)			Ratio sHLA-G/HLA-G$_{EV}$ (ng/ml)		
	$\leq$8.805	>8.805	P value	$\leq$0.850	>0.850	P value	$\leq$4.380	>4.380	P value
Total patients, n (%)	25 (50)	25 (50)	-	25 (50)	25 (50)	-	17 (49)	18 (51)	-
Age at disease diagnosis (median, years)									
>62	9 (36)	16 (64)	**0.047**	10 (40)	15 (60)	0.157	9 (53)	10 (56)	0.876
$\leq$**62**	16 (64)	9 (36)		15 (60)	10 (40)		8 (47)	8 (44)	
Gender									
Males	14 (56)	20 (80)	0.128	17 (68)	17 (68)	>0.999	12 (71)	14 (78)	0.711
Females	11 (44)	5 (20)		8 (32)	8 (32)		5 (29)	4 (22)	
Tumor location									
Rectum	17 (68)	17 (68)	>0.999	18 (72)	16 (64)	0.544	10 (59)	12 (67)	0.631
Colon	8 (32)	8 (32)		7 (28)	9 (36)		7 (41)	6 (33)	
Histological type									
Adenocarcinoma	23 (92)	24 (96)	>0.999	24 (96)	23 (92)	>0.999	16 (94)	16 (89)	>0.999
Others	2 (8)	1 (4)		1 (4)	2 (8)		1 (6)	2 (11)	
Differentiation degree status									
Well+ moderate	22 (96)	23 (96)	>0.999	23 (100)	22 (92)	0.489	15 (94)	15 (94)	>0.999
Poor	1 (4)	1 (4)		0 (0)	2 (8)		1 (6)	1 (6)	
Tumor invasion status (T)									
T1+T2	5 (21)	4 (16)	0.725	6 (25)	3 (12)	0.289	3 (18)	3 (17)	>0.999
T3+T4	19 (79)	21 (84)		18 (75)	22 (88)		14 (82)	15 (83)	
T1+T2+T3	20 (83)	19 (76)	0.725	20 (83)	19 (76)	0.725	12 (71)	15 (83)	0.443
T4	4 (17)	6 (24)		4 (17)	6 (24)		5 (29)	3 (17)	
Regional Lymph Nodes status (N)									
N0	8 (35)	7 (29)	0.679	11 (48)	4 (17)	**0.030**	2 (13)	9 (50)	**0.029**
N+	15 (65)	17 (71)		12 (52)	20 (83)		14 (87)	9 (50)	
Metastasis (M)									
M0	14 (56)	17 (68)	0.382	17 (68)	14 (56)	0.382	8 (47)	13 (72)	0.175
M+	11 (44)	8 (32)		8 (32)	11 (44)		9 (53)	5 (28)	
TNM staging									
I+II	5 (20)	6 (24)	>0.999	8 (32)	3 (12)	0.170	2 (12)	7 (39)	0.121
III+IV	20 (80)	19 (76)		17 (68)	22 (88)		15 (88)	11 (61)	
I+II+III	14 (56)	17 (68)	0.382	17 (68)	14 (56)	0.382	9 (53)	13 (72)	0.305
IV	11 (44)	8 (32)		8 (32)	11 (44)		8 (47)	5 (28)	
Tumor size (cm)									
>4	17 (71)	21 (84)	0.320	18 (75)	20 (80)	0.741	13 (76)	14 (78)	>0.999
$\leq$4	7 (29)	4 (16)		6 (25)	5 (20)		4 (24)	4 (22)	

sHLA-G: soluble HLA-G; EV: extracellular vesicles; Cm: centimeters. Data are represented in numbers (n) and percentages (%). Numbers are estimated from median levels. P values are calculated via the X^2 test or the Fisher's exact test when necessary.

Table 5
Differences in biomarkers production between young, middle-aged and elderly patients participated in our case-control study.

| Molecule (unit)	Young (<40 years)			Middle-aged (40–64 years)			Elderly ($\geq$65 years)		
	Patients (N = 6) Median (range)	Controls (N = 11) Median (range)	P value	Patients (N = 24) Median (range)	Controls (N = 63) Median (range)	P value	Patients (N = 20) Median (range)	Controls (N = 24) Median (range)	P value
sHLA-G (ng/ml)	6.970 (2.46–15.01)	1.446 (0–12.29)	**0.007**	6.600 (0–63)	2.157 (0–63)	**<0.0001**	10.82 (0–21.76)	2.125 (0–4.38)	**<0.0001**
HLA-G$_{EV}$(ng/ml)	1.910 (0–5.69)	0 (0–3.24)	0.112	0.700 (0–17.75)	0.465 (0–31.0)	0.593	1.920 (0–8.59)	0.075 (0–18.39)	**0.012**
IL-6 (pg/ml)	7.320 (2.79–13.70)	4.445 (0–44.12)	0.334	7.135 (0–40.08)	3.660 (0–58.22)	**0.036**	6.190 (0–104.0)	2.225 (0–30.0)	0.230
TNF-α (pg/ml)	15.82 (0–157.5)	0 (0–24.25)	0.086	6.350 (0–90.87)	9.328 (0–218.4)	0.962	0.140 (0–62.50)	13.61 (0–149.7)	**0.009**
IFN-γ (pg/ml)	0 (0–1.15)	0 (0–0)	0.352	0 (0–1.19)	0 (0–356.1)	0.083	0 (0–0.84)	0 (0–357.9)	0.681

sHLA-G: soluble HLA-G; EV: extracellular vesicles; IL: interleukin; IFNγ: interferon-gamma; TNFα: tumor necrosis factor-alpha; n: number. P values calculated using the Mann-Whitney test.

AUC = 0.664 for IL-6. Its higher efficiency to discern CRC population from both CEA and Ca19-9 strengthens the potent role of this cytokine in tumor prediction (Groblewska et al., 2008, Kaminska et al., 2005). Although some articles underlined TNF-α (AUC = 0.880) and IFN-γ (AUC = 0.621) to likely differentiate between CRC and HD specimens (Pengjun et al., 2013), Bunger et al. announced TNF-α (AUC = 0.592) and IFN-γ (AUC = 0.522) as poor predictors (Bünger et al., 2011). Here, we also found that the expression of these cytokines has no utility in CRC identification.

The polarization of T helper1/T helper2 balance towards a Th2 profile was reported as a fundamental mechanism to ensure colorectal tumorgenesis (Kantola et al., 2012). So far, notable evidence stated numerous cytokines to orchestrate HLA-G expression (Persson et al., 2020, Zidi et al., 2006). HLA-G immunosuppressive properties have raised numerous questions about its impact on T lymphocytes proliferation and cytokine secretion. In a prospective study with 122 patients suffering from pancreatic cancer, Xu et al. demonstrated a negative correlation between sHLA-G and T cell
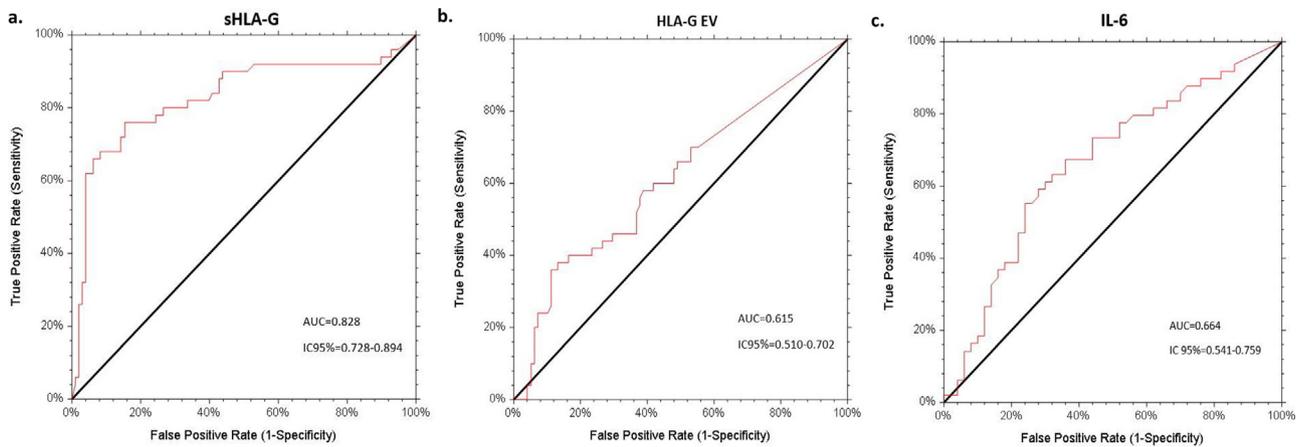
Fig. 3. Receiver operating characteristic (ROC) analyses of sHLA-G, HLA-G$_{EV}$ and IL-6 levels to differentiate between patients and HD groups. The AUCs of sHLA-G, HLA-G$_{EV}$ and IL-6 were 0.828 (95% CI: 0.728–0.894) (a), 0.615 (95% CI: 0.510–0.702) (b) and 0.664 (95% CI: 0.541–0.759) (c) respectively. AUC = area under curve, CI = confidence interval.
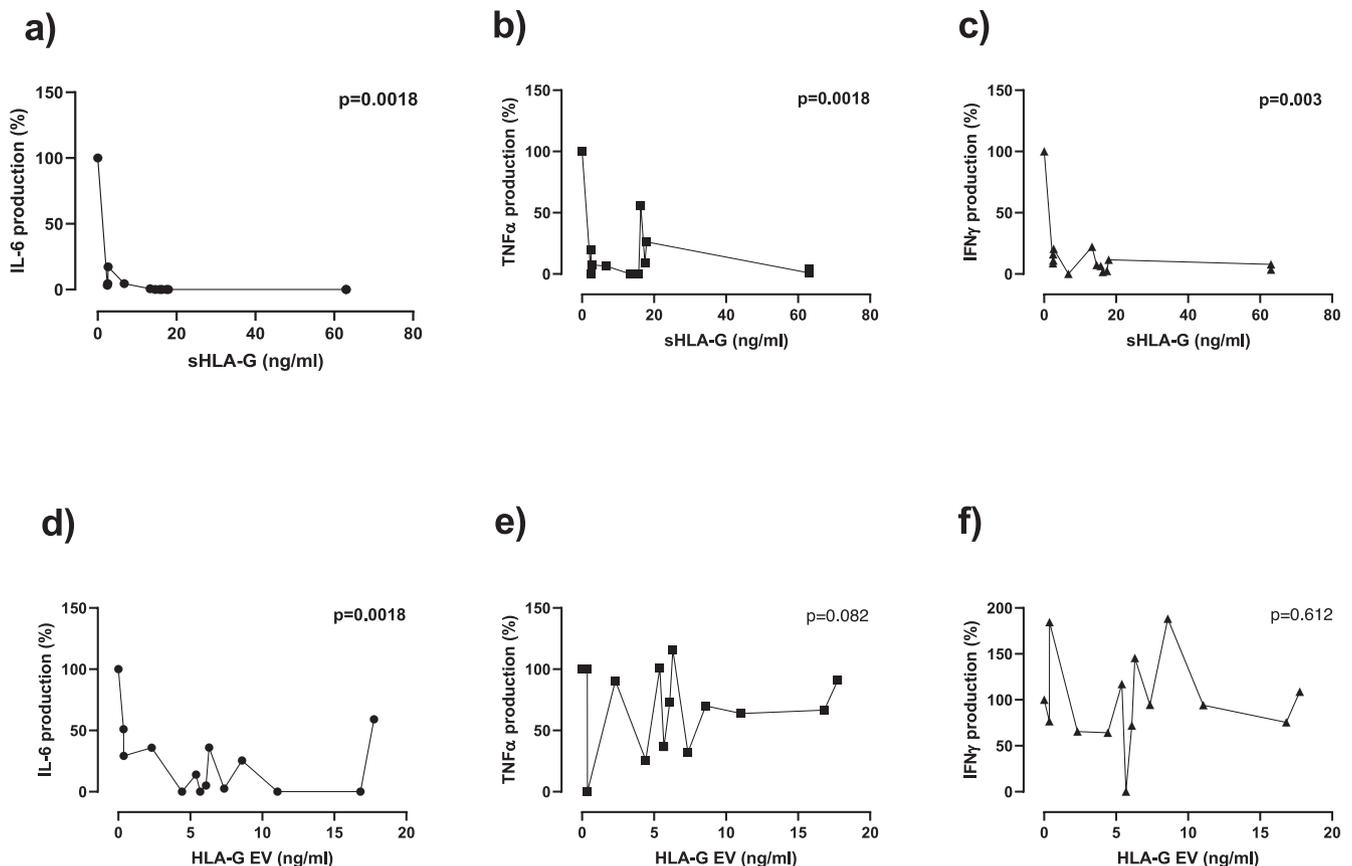
Fig. 4. Cytokine production upon T-cell priming with different amounts of HLA-G. sHLA-G decreases, independently to its concentration, the production of IL-6, TNF-α, and IFN-γ by LT cells (4a-c). HLA-G$_{EV}$ reduced the expression of IL-6 in an independent-dose manner but exhibited no effect on TNF-α and IFN-γ released by T lymphocytes (4d-f). Positive control was used as a reference for comparison.

amounts, which led them to propose a central role of sHLA-G in monitoring lymphocytes T functions as a strategy to restrain immune defense (Xu et al., 2015). Besides, some evidence has shown that the modulation of Th1/Th2 balance depends on certain sHLA-G levels (Kapasi et al., 2000, Maejima et al., 1997). Due to this background, we targeted the potent contribution of HLA-G patterns in triggering T lymphocytes to produce cytokines. Indeed, sHLA-G reduced the production of IL-6, TNF-α, and IFN-γ by activated-T lymphocytes in a concentration-independent manner.

Moreover, HLA-G$_{EV}$ appears to down-modulate the secretion of IL-6 by activated T lymphocytes in a dose-independent manner without such effect on TNF-α and IFN-γ synthesis. This is in alignment with a mild negative correlation found between sHLA-G and IL-6 levels in patients with neurological complications (Almeida et al., 2021). The observed IL-6 decrease by LT cells could be a regulation process to the restoration of the immune system function especially because IL-6 stimulation has been implicated in the restoration of STAT3 in tumor cells (JEG-3) (Liu et al., 2013), in

the development of immune suppressive tolerogenic dendritic cells in vivo (Park et al., 2004b), and in the arrest of maturation/activation of ILT-4 dendritic cells (Liang et al., 2008). Elsewhere, Saurabh and colleagues underlined that HLA-G was antagonistically correlated to TNF-α and IFN-γ in tuberculosis specimens. They additionally proved that blocking HLA-G was sufficient to restore this cytokine production (Saurabh et al., 2018). Another research demonstrated that the injection of HLA-G in mice attenuates IL-6, TNF-α, and IFN-γ expressions (Agaugue et al., 2011). Concomitantly, researchers stated that the amelioration of tumor lesions was associated with Th1 cytokine type in vivo model. It would be crucial to elucidate veridical processes controlling this relation in future studies.

5. Conclusion

In summary, our research has highlighted the importance of both soluble HLA-G forms, sHLA-G and HLA-G$_{EV}$ as potential immune checkpoint molecules to modulate colorectal cancer initiation and progression in the Tunisian population. Based on its impressive performance in colorectal cancer assessment compared to other examined cytokines, sHLA-G might be considered as a more beneficial parameter. These findings strengthen therefore the utility of HLA-G as a diagnosis and a prognostic biomarker in CRC. The potential impact of HLA-G gene polymorphisms on the modulation of its expression should also be discussed in forthcoming studies in order to upgrade our knowledge on genetic-molecular mechanisms during colorectal carcinoma.

CRediT authorship contribution statement

Sabrine Dhouioui: Data curation, Formal analysis, Investigation, Writing original draft. **Nadia Boujelbene:** Validation, Review & Editing. **Hanene Chelbi:** Investigation, Validation. **Ines Zemni:** Validation. **Ines Ben Safta:** Validation. **Hadda-Imene Ouzari:** Validation, Review & Editing. **Amel Mezlini:** Validation. **Abdel Halim Harrath:** Validation, Writing-Review & Editing. **Vera Rebmann:** Methodology, Supervision, Validation, Writing – review & editing. **Inès Zidi:** Conceptualization, Methodology, Supervision, Validation, Writing – review & editing.

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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