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ORIGINAL ARTICLE

Decreased invariant natural killer T-cell-mediated antitumor immune response in patients with gastric cancer

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Abstract

Gastric cancer (GC) is the third most common cause of cancer-related death worldwide. Invariant natural killer T (iNKT) cells are innate-like cytotoxic T lymphocytes involved in tumor immune surveillance. They can be activated either through CD1d-presented glycolipid antigens recognized by their invariant T-cell receptor, cytokines or by sensing tumor-associated stress-induced ligands through the natural killer group 2, member D (NKG2D) receptor. Although the number and functionality of iNKT cells may be decreased in several types of cancer, here we show that GC patients presented a mild increase in iNKT cell frequencies and numbers in the blood compared with healthy donors. In GC patients, iNKT cells, expanded *in vitro* with α-galactosyl ceramide and stimulated with phorbol 12-myristate 13-acetate and ionomycin, produced higher levels of interleukin-2 and transforming growth factor-beta, while their capacity to degranulate remained preserved. Because tumor-derived epithelial cell adhesion molecule-positive epithelial cells did not display surface CD1d, and NKG2D ligands (NKG2DLs) were detected in the gastric tumor milieu, we envisioned a role for NKG2D in iNKT cell functions. Peripheral iNKT cells from GC patients and controls presented similar levels of NKG2D; nevertheless, the percentages of interferon-γ-producing and CD107a-positive iNKT cells from patients were reduced upon challenge with CD1d-negative, NKG2DL-positive K562 cells, suggesting a compromised response by iNKT cells in GC patients, which may not result from impaired NKG2D/NKG2DL signaling. The decreased response of iNKT cells may explain the fact that higher frequencies of circulating iNKT cells did not confer a survival benefit for GC patients. Therefore, functional impairment of iNKT cells in GC may contribute to tumor immune escape and favor disease progression.

INTRODUCTION

Gastric cancer (GC) is the third most common cause of cancer-related death worldwide,[1] with an irregular geographic incidence: high prevalence in Japan, Korea and some regions of Central and South America.[2] In Chile, about 3000 people die annually as a result of GC, representing the most common cause of cancer-related mortality in this country.[3,4] There are multiple ongoing efforts focused on generating new treatments for this

disease, as early diagnosis is rarely carried out in countries without endoscopic screening programs, and GC-associated symptoms are frequently misinterpreted. As a consequence, GC is often detected in advanced stages, which contributes to an overall survival rate as low as 25% after 5 years of gastrectomy.[5] Treatment effectiveness of monoclonal antibody (mAb)-based therapies against GC varies considerably among patients, possibly because of the heterogeneity of this disease and its patient-dependent molecular characteristics.[6] Therefore, immunotherapeutic approaches to treat advanced GC require a deeper understanding of the cellular and molecular mechanisms involving the immune response in this type of cancer.

Invariant natural killer T (iNKT) cells are innate-like T lymphocytes with a semi-invariant T-cell receptor (TCR) that recognizes glycolipid antigens presented by CD1d on antigen-presenting cells (APCs). In humans, the TCR α-chain is restricted to the Vα24-Jα18 rearrangement that pairs to a Vβ11 chain.[7] Upon antigen-dependent activation, iNKT cells can rapidly secrete large amounts of cytokines, including type 1 helper (Th1), Th2 and Th17 cytokines,[8] as well as interleukin (IL)-10,[9] thus amplifying and skewing the immune response toward a specific cytokine secreting profile. Cytokine release by iNKT cells can also be induced by IL-12 and IL-18 in the absence of TCR signaling (reviewed by Reilly *et al.*[10]).

Several reports indicate that iNKT cells are relevant for the orchestration of the initial steps of an adequate antitumor immune response.[11] iNKT cells are able to detect tumor-associated glycolipid antigens *in vivo*[12] and exert CD1d-restricted antitumor effects,[13] as they can directly lyse CD1d-expressing tumor cells using either perforin, granzyme B, TRAIL or Fas ligand.[14,15] The protective role of iNKT cells in antitumor immunity has been revealed by studies showing a selective deficiency in interferon-γ (IFNγ) production and reduced iNKT cell frequencies in the peripheral circulation of patients with diverse types of tumors,[16–22] which, in some cases, have been even correlated with worse prognosis.[23,24] Because various solid tumors do not express CD1d,[25] other mechanisms of iNKT cell recognition of transformed cells may be involved in their antitumor response.

In this sense, the cytokine and cytolytic response of iNKT cells have been reported to be mediated by the natural killer group 2, member D (NKG2D) receptor, an activating NK cell receptor whose ligands expression is induced in conditions of target cell stress and malignant transformation.[26] Increased levels of NKG2D ligands (NKG2DLs) on gastric tumors of small size (≤5 cm) have been associated with better overall survival in patients with gastric adenocarcinoma,[27] which suggests that the immune response against GC may include the activation of iNKT cells by NKG2D. Decreased levels of NKG2D on $CD8^+$ T cells, NK cells and γδ T cells in GC patients have been previously reported, and, in the case of $CD8^+$ T cells, lower levels of NKG2D correlated with decreased production of IFNγ and worse clinical outcome.[28–30] Although NKG2D has been analyzed in the context of GC, the expression and function of this activation receptor on iNKT cells of GC patients have not been revised.

In this study, we evaluated the frequency and absolute numbers of iNKT cells in the peripheral blood of GC patients and healthy donors (HDs), as well as the surface levels of NKG2D on these effector cells. Peripheral iNKT cells from GC patients and controls were expanded *in vitro* and purified in order to analyze their cytokine production and cytolytic activation upon challenge with a CD1d-negative, NKG2DL-positive tumor cell line. Our results provide evidence that GC patients presented a mild increase in the frequency and numbers of peripheral iNKT cells, which displayed similar levels of NKG2D. However, the compromised functionality of iNKT cells observed in GC patients may at least partially explain the lack of correlation between higher iNKT cell frequency and patient overall survival, suggesting that deficiency in iNKT cell-mediated antitumor immunity may contribute to tumor progression in patients with gastric adenocarcinoma.

RESULTS

Frequencies and numbers of peripheral iNKT cells in GC patients

The percentages and absolute numbers of peripheral iNKT cells in the peripheral blood mononuclear cells (PBMCs) of 21 GC patients and 23 HD were analyzed by flow cytometry (Figure 1a). iNKT cells were identified as $CD3^+$TCR Vα24-J$\alpha18^+$TCR $V\beta11^+$ lymphocytes (complete gating strategy presented in Supplementary figure 1). Peripheral iNKT cell frequencies in controls and GC patients were (percentage of mean value ± standard deviation) 0.28 ± 0.2 and 0.62 ± 0.57, respectively. A mild increment in the frequency of these cells in GC patients could be detected, as compared with HDs (Figure 1b). Similarly, a moderate increase in the numbers of iNKT cells per milliliter of blood was found in GC patients (Figure 1c). We next analyzed the survival rate of GC patients after potentially curative gastrectomy. Because a variable frequency of iNKT cells was observed among patients, we decided to define two groups of patients based on the median value of the percentages of iNKT cells obtained for all patients analyzed. One group was composed of patients with normal iNKT cell frequency (0.21 ± 0.13, $n = 10$), whereas the other group presented higher frequency of these cells in the blood (0.99 ± 0.55,

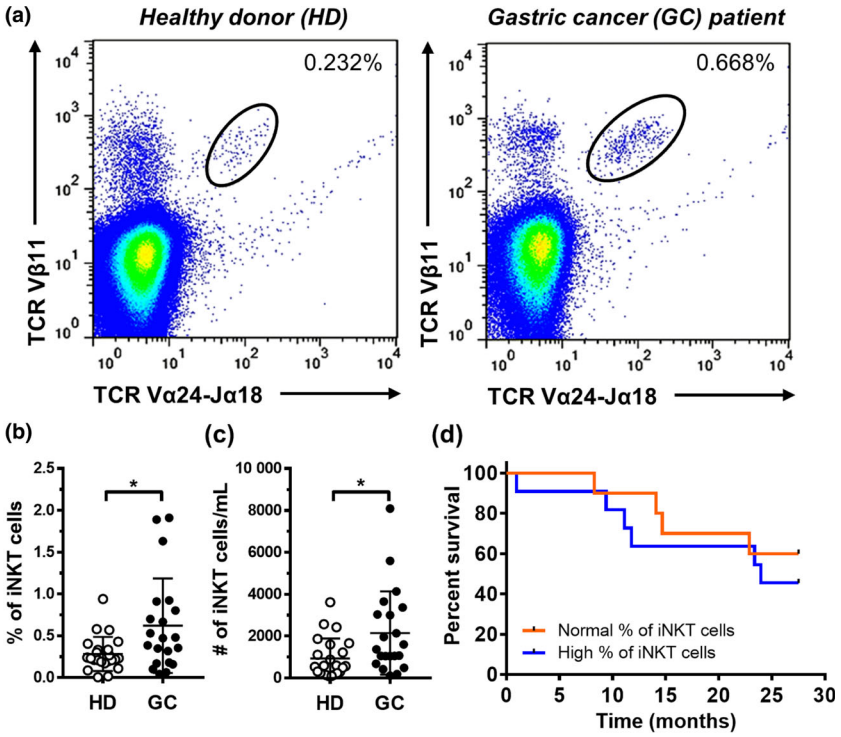

Figure 1. The frequency of invariant natural killer T (iNKT) cells in the peripheral blood of gastric cancer (GC) patients does not correlate with patient overall survival rate after gastrectomy. **(a)** Representative flow cytometry dot plots of peripheral blood mononuclear cells (PBMCs) of a healthy donor (HD) and a GC patient showing the percentages of T-cell receptor (TCR) Vα24Jα18$^+$TCR Vβ11$^+$iNKT cells from total CD3$^+$ lymphocytes. The gating strategy used to select iNKT cells in the PBMC is shown in Supplementary figure 1. The **(b)** frequencies and **(c)** absolute numbers of peripheral iNKT cells per milliliter of blood were analyzed; bars represent the mean values ± s.d. obtained from the analysis of 21 GC patients and 23 HDs. Dots indicate independent donors, and experiments were performed once for each donor within each group. A Mann–Whitney *U*-test was used to analyze differences between groups (*$P < 0.05$). **(d)** Kaplan–Meier survival curves for GC patient overall survival, after gastrectomy, according to their normal ($n = 10$) and high ($n = 11$) percentages of peripheral iNKT cells. Group definition was based on the median value of the percentages of iNKT cells observed on PBMC samples from all 21 GC patients analyzed. Significance was determined by the log-rank test. s.d., standard deviation.

$n = 11$). A follow-up period of 28 months after surgery revealed that high, compared with normal iNKT cell frequency could not be associated with better overall survival rate of patients (Figure 1d), suggesting that increased levels of iNKT cells in the peripheral blood do not guarantee better survival for GC patients. Accordingly, iNKT cell frequency did not associate with clinical–pathological features of tumors (Table 1; data not shown).

Cytokine production by iNKT cells from GC patients

It has been well established that cytokines produced by iNKT cells are important to determine the type and magnitude of the immune response to tumors.[31] Thus, we decided to examine the cytokine profile of peripheral iNKT cells in GC patients. As any antigen-specific T-cell population, iNKT cells represent a small cell population in human blood, which poses some limitations for direct *ex vivo* functional analysis of these cells. Therefore, we cultured PBMC for 4 weeks in the presence of IL-2 and IL-7 plus a single initial pulse with the strong iNKT cell agonist α-galactosyl ceramide (α-GalCer) to induce iNKT cell enrichment (Figure 2a).

The phenotyping of postexpanded CD3$^+$ cells revealed two cell subpopulations expressing iNKT cell-specific α and β TCR chains: a Vα24-Jα18$^+$Vβ11^{int} subset and a prominent population with high expression of both Vα24-Jα18 and Vβ11 TCR chains. We decided to purify exclusively this latter cell population, because it was the well-defined and characteristic iNKT cell phenotype detected in the PBMC of both patients and controls (Figure 1a). Therefore, iNKT cells were sorted according to the expression of CD3 and high expression of iNKT cell-restricted TCR chains (Figure 2a and Supplementary figure 1). Purified iNKT cells were then stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, after which intracellular cytokine production was examined by flow cytometry.

Table 1. Demographic and clinical–pathological information of gastric cancer patients

Variables	*n* (%)
Sex	
Male	33 (67)
Female	16 (33)
Age at surgery (years)	
Mean (range)	65 (41–90)
Tumor size (cm)	
≤5	16 (34)
>5	31 (66)
Tumor differentiation	
High/medium	15 (33)
Low/none	31 (67)
Invasion status[a]	
T1, T2	13 (28)
T3, T4	34 (72)
Lymph node metastasis (*N*)	
No (N0)	13 (27)
Yes (N1, 2 ,3)	35 (73)
TNM stage[b]	
I, II	16 (34)
III, IV	31 (66)

[a]Invasion status is defined by tumor invasion in the epithelium or mucosa of the lamina propria, muscularis mucosae or submucosa (T1), muscularis propria or subserosa (T2), serosa (T3) or adjacent organs (T4).
[b]TNM stages for gastric cancer were defined according to the classification of the American Joint Committee on Cancer.[42] Patients' data were provided by the Pathology Department of Hospital del Salvador (Santiago, Chile).

On the one hand, we observed that pharmacological stimulation of iNKT cells from controls induced the production of IL-2, IFNγ, transforming growth factor-beta (TGF-β) and, to a lesser extent, IL-10 by these cells (Figure 2b). On the other hand, the percentages of GC patients' iNKT cells producing these cytokines did not change upon PMA and ionomycin stimulus (Figure 2b). However, the proportions of iNKT cells from GC patients producing IL-2 and TGF-β were significantly higher than those from controls, either in the absence or in the presence of PMA and ionomycin, while the percentages of iNKT cells producing IFNγ and IL-10 remained similar between patients and controls, regardless of stimulus (Figure 2b). These results reveal an intrinsic ability of iNKT cells from GC patients to produce higher levels of IL-2 and TGF-β upon antigenic stimulus with α-GalCer, as compared with controls.

iNKT cells from GC patients are capable of degranulating

Because iNKT cells are cytolytic lymphocytes that can directly lyse tumor cells, we decided to evaluate the expression of the cytolytic degranulation marker CD107a on expanded and purified iNKT cells after stimulus with PMA and ionomycin. Pharmacological treatment induced an increase in the percentages of iNKT cells expressing CD107a in both GC patients and HD (Figure 2c). Interestingly, GC patients and HDs presented similar percentages of CD107a-positive iNKT cells in the absence or presence of stimulus (Figure 2c), indicating that expanded and purified iNKT cells from GC patients maintain the potential for cytolytic degranulation.

Epithelial cell adhesion molecule$^+$ epithelial cells in the gastric tumor do not express CD1d

Because TCR stimulation by CD1d-positive tumor cells has been shown to trigger iNKT cell activation,[10] we decided to explore the possibility of canonical CD1d-mediated response of iNKT cells to gastric adenocarcinoma cells. Total mRNA was extracted from primary gastric adenocarcinoma samples and adjacent nontumor gastric mucosa tissues from GC patients and the expression of the *cd1d* gene was examined by real-time PCR. Tumor samples presented a twofold higher expression of the *cd1d* gene compared with gastric mucosa (Figure 3a). We next evaluated whether CD1d is expressed by epithelial cells in the tumor microenvironment. For this purpose, gastric tumor and mucosa samples were dissociated into single-cell suspensions; stained with fluorescence-conjugated antihuman CD1d, epithelial cell adhesion molecule (EpCAM) and CD19 mAbs and analyzed by flow cytometry. Tumor-infiltrating CD19-positive B cells displayed CD1d on the cell surface, but this molecule could not be detected on EpCAM-positive epithelial cells (Figures 3b, c). Therefore, our results show that primary gastric adenocarcinoma cells lack CD1d expression and that iNKT cell direct response to these tumor cells may not involve TCR signaling.

NKG2D ligands are expressed on gastric adenocarcinoma tissue cells

As in the case for NK cells, CD8$^+$ T cells and γδ T lymphocytes, iNKT cells have also been shown to express NKG2D.[32] This cytotoxicity-associated activating receptor binds to a wide range of ligands (NKG2DL) expressed by target cells, which are upregulated in conditions of cell stress and malignant transformation. Recognition of NKG2DL on the surface of tumor cells triggers the release of preformed granules containing perforin and granzymes by effector cells, resulting in direct tumor cell lysis.[33] NKG2DL comprises major histocompatibility complex class I-related chains A and B (MICA and MICB) and a family of six UL16-binding proteins

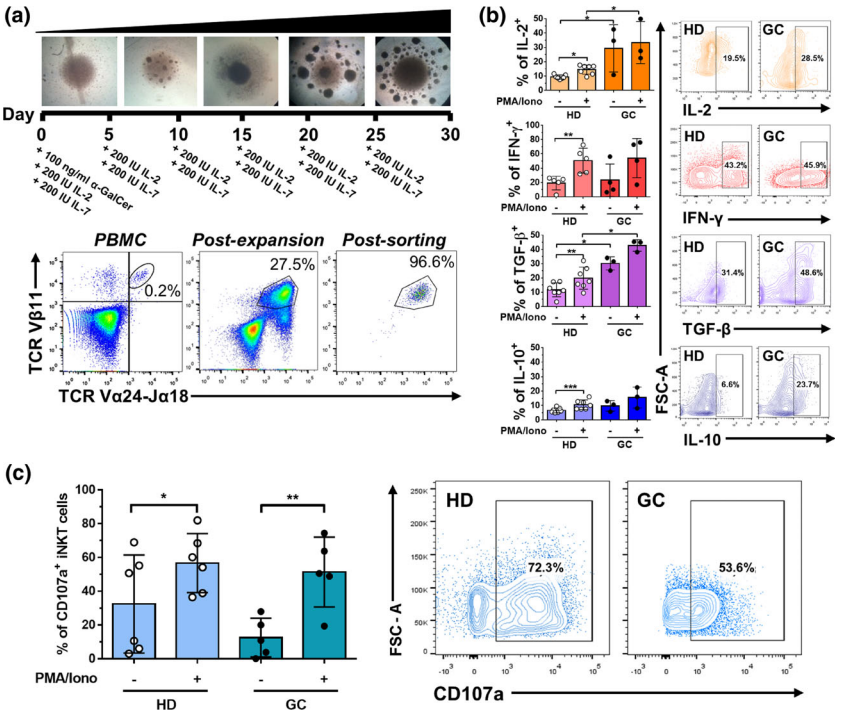

Figure 2. Gastric cancer (GC) patients present higher proportions of interleukin-2 (IL-2)- and transforming growth factor-beta (TGF-β)-producing invariant natural killer T (iNKT) cells and maintain the potential for cytolytic degranulation. **(a)** Protocol for *in vitro* iNKT cell expansion and purification. Peripheral blood mononuclear cell (PBMC) from GC patients and controls were used to expand iNKT cells using a single pulse of α-galactosyl ceramide (α-GalCer); cells were maintained in iNKT cell medium containing IL-2 and IL-7 for 4 weeks. Representative images show cell culture evolution of PBMC from a GC patient. Expanded iNKT cells were stained using specific monoclonal antibodies (mAbs) and purified by cell sorting. Representative dot plots of T-cell receptor (TCR) Vα24-Jα18$^+$ and TCR Vβ11$^+$ cells from total CD3$^+$ lymphocytes present in the initial PBMC, 30 days after *in vitro* expansion, and after cell sorting are shown. The percentages of iNKT cells are indicated in the dot plots for each condition. **(b)** Expanded and purified iNKT cells from GC patients (n = 3 or 4) and healthy donors (HDs; n = 5 or 7) were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (PMA/Iono) for 5 h plus brefeldin A and monensin, fixed and permeabilized for intracellular cytokine staining and analyzed by flow cytometry using mAbs to iNKT cells and specific cytokines. To the left, all bars represent mean values ± s.d. obtained from one measurement for each donor within each group. A Mann–Whitney *U*-test or an unpaired *t*-test was carried out to analyze differences between groups (*P < 0.05, **P < 0.01, ***P < 0.001). To the right, representative dot plots of PMA/Iono-stimulated iNKT cells from a HD and a GC patient are shown. **(c)** Expanded and purified iNKT cells from GC patients (n = 5) and HD (n = 6) were stimulated with PMA/Iono for 5 h in the presence of anti-CD107a APC-Cy7 mAb plus brefeldin A and monensin, followed by analysis by flow cytometry using mAbs to iNKT cells. To the left, all bars represent mean values ± s.d. obtained from one measurement for each donor within each group. An unpaired *t*-test was used to evaluate differences between groups (*P < 0.05, **P < 0.01). To the right, representative dot plots of PMA/Iono-stimulated iNKT cells from a HD and a GC patient are shown. FSC, forward scatter; IFNγ, interferon-gamma; s.d., standard deviation.

(ULBP1–6).[34] Expression of at least one NKG2DL has been shown in most human epithelial cancers,[35] including GC.[36]

To further examine the alternative activation of iNKT cells through NKG2D, we decided to evaluate the surface expression of NKG2DL on cells present in the primary gastric adenocarcinoma tissue by flow cytometry. All NKG2DLs evaluated, which included MICA, MICB, ULBP-1, ULBP-2/5/6 and ULBP-3, were detectable in the gastric tumor and mucosal tissues of GC patients (Table 2 and Supplementary figure 2), suggesting that these tumors may become targets of iNKT cell response mediated by NKG2D.

iNKT cells from GC patients and HD have similar levels of NKG2D

Next, we analyzed the cell surface expression of NKG2D on peripheral blood iNKT cells. The gating strategy for flow cytometry analysis of NKG2D levels is shown in Supplementary figure 1. Approximately 48% of circulating iNKT cells from patients and controls expressed NKG2D (Figure 4a). Interestingly, no statistically significant difference in the levels of this receptor was observed between iNKT cells from patients and HD (Figure 4b). Of note, the levels of NKG2D on iNKT cells from patients and controls tended to increase

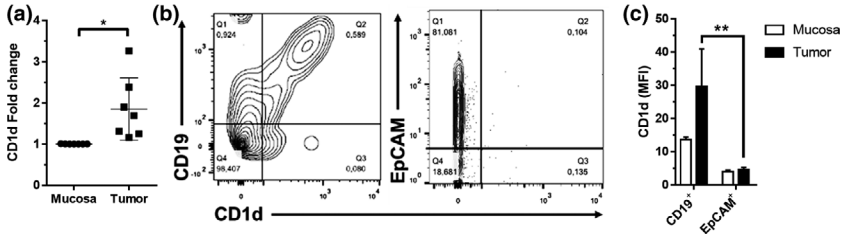

Figure 3. Detection of CD1d expression in the gastric tumor microenvironment. **(a)** The relative expression of the *cd1d* gene in the primary tumor and adjacent gastric mucosa of seven gastric cancer (GC) patients was quantified by real-time PCR. Expression values were calculated using the ΔΔCt method and expressed as the fold change of the messenger RNA levels of *cd1d* in the gastric tumor relative to mucosal samples. *Hprt* expression was used as the housekeeping control. Bars represent mean values ± s.d. obtained from PCRs performed in triplicates for each donor within each group of gastric tissue. An unpaired *t*-test was used to assess differences between groups (*$P < 0.05$). **(b)** Representative flow cytometric contour plots of dissociated cells from the gastric tumor of a GC patient showing the analysis of cell surface expression of CD1d in tissue-infiltrating $CD19^+$ B cells and epithelial cell adhesion molecule $(EpCAM)^+$ epithelial cells. **(c)** The graph shows the CD1d levels, expressed as median fluorescence intensity values, on $CD19^+$ B cells and $EpCAM^+$ epithelial cells in the gastric tumor and surrounding mucosa from six GC patients. Bars represent mean values ± s.d. obtained from one measurement for each donor within each group of gastric tissue. An unpaired *t*-test was used to evaluate differences between groups (**$P < 0.01$). s.d., standard deviation.

Table 2. Expression levels of natural killer group 2, member D ligands (NKG2DLs) on cells derived from the primary gastric tumor and nontumor mucosa tissues of GC patients

NKG2DL (MFI ± s.d.)	MICA	MICB	ULBP-1	ULBP-2/5/6	ULBP-3	IgG2a
Tumor	41.54 ± 74.83	39.38 ± 75.46	112.5 ± 204.4	78.36 ± 140.6	112.7 ± 239.8	5.45 ± 3.72
Mucosa	33.7 ± 60.62	23.25 ± 31.62	72.99 ± 143.1	60.76 ± 104.7	85.05 ± 172.2	5.82 ± 3.40

Mean values ± standard deviation (s.d.) of the median fluorescent intensity (MFI) values obtained from 21 gastric cancer (GC) patients for all ligands (except ULBP-2, $n = 18$) detected in gastric adenocarcinoma and mucosa-derived cells by flow cytometry.

after iNKT cell expansion and purification, although no statistically significant difference could be detected (Figure 4c). Therefore, because iNKT cells from GC patients express CD107a and NKG2D at levels similar to those from controls, we hypothesized that these cells may be able to target tumor cells through a cytotoxic response involving the NKG2D–NKG2DL axis.

iNKT cells from GC patients present a compromised antitumor response

To examine whether iNKT cells can degranulate and produce IFNγ after challenge with a tumor cell line, we incubated expanded and purified iNKT cells with K562 cells and evaluated CD107a expression and IFNγ production by iNKT cells. Although K562 cells are commonly used in human NK cells cytotoxic assays, as they do not express major histocompatibility complex class I, these tumor cells can also be used to assess the alternative activation of iNKT cells mediated by NKG2D, because they present high levels of NKG2DL and do not express CD1d[32] (Supplementary figure 3). After 5 h of effector and target cell coculture, cells were retrieved, stained and analyzed by flow cytometry. We found significantly lower percentages of $CD107a^+$ iNKT cells from GC patients in comparison with HDs when using 1:1 effector-to-target cell ratio (Figure 5a), whereas the percentages of iNKT cells producing IFNγ were significantly decreased in GC patients as compared with HDs at all effector-to-target cell ratios (Figure 5b). Interestingly, the levels of NKG2D were variable in both patients and controls at all effector-to-target cell ratios analyzed, with no significant differences found between the mean values on each group (Figure 5c). MKN-45 gastric adenocarcinoma cells could also trigger cytolytic activation and IFNγ production by iNKT cells from an HD, whereas such response was lower in iNKT cells from a GC patient (data not shown). Altogether, our data indicate that, in GC patients, even though PMA and ionomycin promote CD107a expression on iNKT cells, and that these cells display normal levels of NKG2D, their capacity to degranulate and produce IFNγ upon challenge with a target tumor cell that lacks CD1d but expresses NKG2DL is impaired.

DISCUSSION

iNKT cells are among the immunity's first responders to infection, autoimmune diseases and other disorders,[8] as

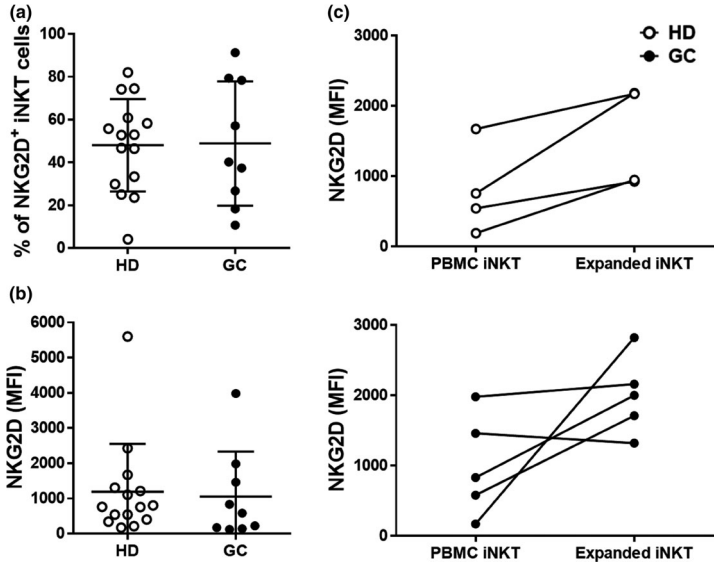

Figure 4. Invariant natural killer T (iNKT) cells from gastric cancer (GC) patients and healthy donors (HDs) present similar levels of natural killer group 2, member D (NKG2D). The gating strategy to evaluate, by flow cytometry, the expression of NKG2D on peripheral blood iNKT cells from GC patients and HDs is shown in Supplementary figure 1. **(a)** The percentages of iNKT cells expressing NKG2D and **(b)** the levels of this receptor [in median fluorescence intensity (MFI) values] on iNKT cells were evaluated on 9 GC patients and 15 HDs. An unpaired *t*-test or a Mann–Whitney *U*-test was performed to analyze differences between groups. Bars represent mean values ± s.d. obtained from one measurement for each donor within each group. **(c)** The levels of NKG2D on iNKT cells from five GC patients and four HDs were assessed before and after iNKT cell expansion. Wilcoxon or paired *t*-tests were used for statistical analysis. s.d., standard deviation.

they do not require priming, rapidly produce IFNγ, express CD40L[37] and present similar levels of IL-12 receptor compared with NK cells.[38] However, iNKT cells exhibit an impaired functionality or their numbers are reduced in the blood of cancer patients,[39] suggesting that they are important players of the antitumor immunity. Here, we show that, although the percentages of iNKT cells in the blood of GC patients are preserved, or even increased in some patients, and their functionality is maintained after *in vitro* expansion in the presence of α-GalCer, these innate-like lymphocytes are less sensitive to a CD1d-negative, NKG2DL-positive tumor cell line. The defective functional phenotype of iNKT cells from GC patients implies a compromised immune response that may favor tumor progression.

Only few studies have evaluated the role of iNKT cells in GC. In one report, Peng *et al.* showed that the frequencies of a population of circulating $CD3^+CD56^+$ T lymphocytes, which they named NKT-like cells, were similar between GC patients and controls.[40] Notably, Kelly-Rogers *et al.* observed that approximately 50% of peripheral $CD3^+CD56^+$ T cells in healthy individuals are $CD8^+$ T lymphocytes.[41] Therefore, it is possible that the subset of NKT-like cells analyzed by Peng *et al.* in GC patients and controls included both iNKT and $CD8^+$ T lymphocytes, among other subpopulations of T cells that constitutively express CD56. Our work, alternatively, proposed a selective analysis of iNKT cells making use of their characteristic TCR chains. We found an unexpected mild increase in iNKT cell frequencies and numbers in the peripheral blood of GC patients compared with healthy individuals. Interestingly, patients' demographic data, such as age and sex, and clinical–pathological parameters of the disease, including tumor size, differentiation and extension, the percentage of lymph node metastasis and the tumor, node and metastasis stage[42] did not associate with iNKT cell levels (data not shown). Accordingly, GC patients presenting with increased frequencies of peripheral iNKT cells at surgery showed a similar overall survival rate compared with that of patients with normal levels of iNKT cells, indicating that higher frequency of iNKT cells in the blood does not promote a better prognosis for the disease.

It is well established that, upon activation, iNKT cells can secrete a large spectrum of Th1, Th2 and Th17 cytokines, thus enhancing host immunity to microorganisms and cancer.[8] By contrast, various studies have demonstrated a decrease in IFNγ production by iNKT cells in cancer patients.[18,20] Recently, a new subset of IL-10-producing iNKT cells has been described,[9] and some researchers have suggested a regulatory role for these cells in the context of cancer.[43] Therefore, analyzing

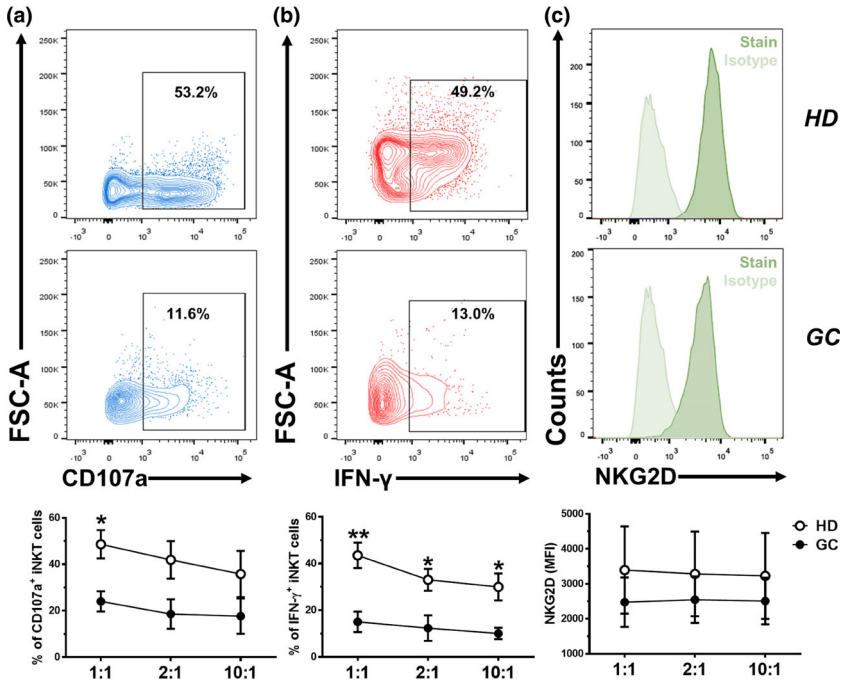

Figure 5. Invariant natural killer T (iNKT) cells from gastric cancer (GC) patients present compromised degranulation capacity and interferon-gamma (IFNγ) production against K562 target cells. Purified and expanded iNKT cells from four GC patients and four healthy donors (HDs) were cocultured with K562 cells for 5 h at different effector-to-target (E:T) cell ratios. CD107a, IFNγ and natural killer group 2, member D (NKG2D) were evaluated by flow cytometry. **(a)** Representative dot plots of CD107a expression on iNKT cells from an HD (top) and a GC patient (middle). At the bottom, the graph shows accumulated data of the percentages of $CD107a^+$ iNKT cells from HDs (open circles) and GC patients (black circles) at different E:T cell ratios. **(b)** Representative dot plots of intracellular IFNγ production by iNKT cells from an HD (top) and a GC patient (middle); bottom, accumulated data of the percentages of $IFN\gamma^+$ iNKT cells from HD and GC patients at different E:T cell ratios. **(c)** Representative histograms of NKG2D levels on iNKT cells from an HD (top) and a GC patient (middle). Light green histograms, isotype control; green histograms, NKG2D staining. Bottom, accumulated data of the median fluorescence intensity (MFI) values for NKG2D levels on iNKT cells from HD and GC patients at different E:T cell ratios. For all accumulated data, bars represent mean values ± s.e.m. Results are from independent experiments, one measurement for each donor performed in triplicate wells for each E:T ratio. Multiple *t*-tests were carried out to evaluate differences between groups (*$P < 0.05$, **$P < 0.01$). FSC, forward scatter; s.e.m., standard error of the mean.

the cytokine profile of these cells in GC patients gains relevance. We observed that, after expansion, iNKT cells from GC patients produced IL-2 and TGF-β at higher proportions compared with HD, whereas the percentages of IFNγ- and IL-10-producing iNKT cells remained similar between patients and controls. This cytokine profile was detected upon exogenous antigenic (α-GalCer) and growth factors (IL-2 and IL-7) stimulation alone (for cell expansion settings), or under the same conditions followed by PMA and ionomycin stimulus. Based on these results, we propose that the relatively increased levels of IL-2 and TGF-β produced by iNKT cells could be favoring the expansion of regulatory T cells in GC patients.[44] TGF-β is well known to transform naïve T cells into peripheral induced regulatory T cells.[45] Because TGF-β-driven conversion of regulatory T cells has been observed in GC patients,[46] it is possible that higher levels of IL-2 and TGF-β from iNKT cells may, at least in part, account for the increased proportions of regulatory T cells in these patients. However, it is worth mentioning that, here, we also detected a marked heterogenous response in the proportions of iNKT cells producing IL-2, IFNγ and IL-10, while some consistency was observed for TGF-β production. This may be a result of the interindividual variability often observed in human studies and/or the prevalence of iNKT cell subsets producing different types of cytokines in individual patients, which may influence the immune response to this type of cancer. Hence, it is clearly necessary to increase patient sample size to obtain more powerful results, which can be relevant for future iNKT cell-targeted therapies in GC.

Based on the fact that iNKT cell activation can be mediated through either TCR signaling, NK cell receptor-dependent pathways or cytokines alone,[10] it is important to take into consideration that the conditions used in our assays to favor iNKT cell expansion from fresh blood samples, which included α-GalCer and recombinant IL-2 and IL-7 stimulation, may have affected the phenotype

and functions of these cells. Indeed, it has been demonstrated that IL-7 effectively supports human iNKT cell proliferation *in vitro*,[31] preferentially promoting $CD4^+$ iNKT cell proliferation and inducing IFNγ and IL-4 production in a fraction of neonatal human iNKT cells.[47] Therefore, the use of lipid-antigen stimulation and/or addition of IL-2 and IL-7 into the culture medium of expanding iNKT cells may explain, at least in our settings, the relatively high baseline production of cytokines observed in both patients and controls. Accordingly, the number and functions of iNKT cells that infiltrate the gastric tumor milieu may be influenced by soluble factors and cytokines produced by transformed epithelial cells and tumor-infiltrating immune cells,[48] thus determining their response to solid tumors. For instance, Peng *et al.* reported decreased percentages of tumor-infiltrating $CD3^+CD56^+$ NKT-like cells, which presented decreased IFNγ, TNF-α, granzyme B and Ki-67 expression, as well as diminished CD69, NKG2D and DNAM-1 surface levels as compared with nontumor gastric mucosa. These authors also showed that decreased frequencies of tumor-infiltrating NKT-like cells with functional impairment correlated with tumor progression and poor survival of GC patients. They also demonstrated that soluble factors released by gastric tumor tissue induced a decrease in the production of IFNγ, TNF-α, granzyme B and Ki-67 by peripheral NKT-like cells from healthy controls.[40] These data suggest that the effector functions of NKT-like cells may be modulated by soluble factors secreted by GC tumors. Thus, further investigation is needed to uncover if this is also the case for tumor-infiltrating iNKT cells yet considering that classical activation of iNKT cells through TCR recognition of glycolipid antigens presented by CD1d may also occur at the tumor site.

In this sense, here we showed that primary gastric adenocarcinoma samples presented increased levels of *cd1d* mRNA as compared with gastric mucosa. Interestingly, CD1d was detected on $CD19^+$ B cells, but not on EpCAM-positive epithelial tumor cells. It has been suggested that one of the potentials of iNKT cell-mediated antitumor response is to eliminate $CD1d^+$ tumor-associated monocytes/macrophages and/or $CD1d^+$ myeloid-derived suppressor cells in the microenvironment of CD1d-negative tumors, both of which have been shown to contribute to tumor progression.[25] Even though GC seems to be a CD1d-negative tumor, tumor-associated APCs expressing CD1d are present in gastric adenocarcinomas, which could promote an indirect immune response mediated by iNKT cells.[49] Thus, we cannot exclude the possibility that tumor-infiltrating APCs, such as $CD19^+CD1d^+$ B cells, modulate iNKT cell response to tumors and influence the characteristics of iNKT cells described for GC patients. Indeed, B cells may aid tumor immune escape by secreting soluble immune-regulating factors. For instance, increased frequencies of B cells producing IL-35, an immune-regulatory cytokine, have been reported in untreated GC patients, which was associated with GC progression.[50] Hence, an attractive line of investigation emerges, because regulatory B cells have been proposed to maintain iNKT cells homeostasis in humans.[51] Therefore, colocalization of iNKT cells along with B lymphocytes and other myeloid cells in specific tumor niches may reveal important information to better define the *in situ* functions of iNKT cells in this disease. Because PMA and ionomycin stimulus promoted degranulation and IFNγ production by patients' iNKT cells, it would be interesting to evaluate whether iNKT cell-mediated cytokine secretion and cytotoxicity can be activated through TCR engagement after challenge with $CD1d^+$ APCs or CD1d-transfected target tumor cells pulsed with α-GalCer. Still, cytolytic activation of iNKT cells has been reported with α-GalCer in the absence of CD1d on target cells,[8,14] although this is a high-affinity antigen with few similarities to low-affinity tumor antigens so far described.[52] In addition, immune-regulatory tumor-associated antigens recognized by iNKT cells have been described for other types of cancer,[53] but not for GC.

iNKT cells can also target tumor cells through NKG2D. Because NKG2DLs were detected on cells present in the primary gastric adenocarcinoma, we decided to evaluate the levels of NKG2D on circulating iNKT cells. Contrary to what has been reported for other peripheral cytolytic cells in GC patients, such as $CD8^+$ T cells, whose decreased NKG2D levels correlated with a poorer clinical outcome,[54] we observed similar levels of NKG2D on iNKT cells between patients and controls. However, when challenged with K562 cells, a tumor cell line that does not express CD1d but displays NKG2DL on the cell membrane, the percentages of iNKT cells expressing CD107a and producing IFNγ were significantly lower in GC patients compared with controls, whereas the levels of NKG2D on iNKT cells were comparable between both groups after co-incubation with tumor cells. Therefore, our results suggest that degranulation and IFNγ production by iNKT cells from GC patients are impaired upon challenge with a target tumor cell line that expresses NKG2DL and lacks CD1d. It is possible that NKG2D downstream signaling pathways are compromised in iNKT cells from GC patients. NKG2D activation can act synergistically but also independently from other activating pathways, including TCR signaling[32] thus, downregulation of specific signaling kinases or adaptor proteins could be compromising tumor-induced activation of iNKT cells in the absence of TCR stimulation.[55] By contrast, our patient data on spontaneous expression of CD107a and IFNγ production

by iNKT cells, which were similar to those obtained upon target cell challenge, suggest that iNKT cell-mediated cytokine production and degranulation do not depend on NKG2D signaling. Alternatively, cytokine production and cytolytic activation of iNKT cells may be mediated by other activating receptors that are also present on iNKT cells, such as DNAM-1,[32] whose expression levels and/or functions may be reduced in GC. Another possibility is that decreased CD107 levels and IFNγ production by iNKT cells from GC patients, associated with target cell challenge, may result from the activation of inhibitory mechanisms, such as those mediated by PD-1,[56] Tim-3,[57] LAG-3[58] and/or CD94/NKG2A.[59]

The numbers of cell-based treatments for cancer have increased sharply in recent years, such as those using chimeric-antigen receptor-transfected T cells. iNKT cells have been considered a new specialized host for transfection, as longer persistence and increased survival of chimeric-antigen receptor-iNKT cells *in vivo* have been reported.[60] In addition, α-GalCer intravenous administration is safe in humans,[61] and a mouse model for allogenic adoptive transfer of chimeric-antigen receptor-iNKT cells instead of chimeric-antigen receptor-T cells has proven not to produce graft-*versus*-host disease.[60] In this regard, an important consideration about our results is that GC patients present iNKT cells that, upon *in vitro* expansion, display a compromised antitumor response, with lower production of IFNγ and less degranulation capacity toward tumor cells. These data imply that even though peripheral iNKT cells from patients were pulsed with α-GalCer and expanded in the presence of the Th1-promoting cytokine IL-7, such experimental conditions could not reverse the defects shown by these cells. These findings are particularly interesting, as multiple ongoing clinical trials use autologous expanded iNKT cells for adoptive transfer to cancer patients.[62,63] As reversing iNKT cell-deficient response in cancer is already a goal of multiple groups working on clinical trials targeting iNKT cells,[49] we believe that correction of iNKT cell defects in GC should also be a concern when testing their efficacy in cellular therapies against this disease.

METHODS

Patients

A total of 49 patients, 16 females and 33 males, aged 65 ± 11.5 years (range, 49–82), pathologically diagnosed with gastric adenocarcinoma, were recruited at the Department of Surgery (Oriente), Hospital del Salvador (Santiago, Chile). Blood samples from 27 HD, 14 females and 9 males, aged 50.7 ± 8 years (range, 38–64), with no history of malignant disease, were obtained for this study. All HDs and patients signed a written informed consent for blood and gastric tissue samples donation before surgery. Patients who received chemotherapy or radiotherapy for GC treatment before gastrectomy were not included in this study. Primary gastric tumor and adjacent nonmalignant gastric mucosa samples were collected immediately after surgical resection of the stomach. Demographic and clinical–pathological information of recruited patients is summarized in Table 1. Clinical–pathological features of tumors were determined according to the disease staging system of the American Joint Committee on Cancer.[42] Histopathological diagnosis was performed by pathologists from Hospital del Salvador. Patients' survival was assessed during 28 months after potentially curative surgery or until death as a result tumor-specific disease. All protocols were approved by the Committee on Human Ethical Investigation of the School of Medicine, University of Chile, and the Committee on Scientific Ethics of the Metropolitan Health Service of the Chilean Government. All experiments were performed in accordance with our institutional guidelines and with the Declaration of Helsinki.

Blood and gastric tissue samples preparation

Blood samples from GC patients and HDs were obtained in heparinized tubes, and PBMCs were prepared by standard procedures. In brief, blood samples were diluted in phosphate-buffered saline (PBS) and centrifuged on a Ficoll-Paque (GE Healthcare, Chicago, IL, USA) gradient to separate PBMC. Cells were washed with PBS and cryopreserved in liquid nitrogen in fetal bovine serum (FBS; Corning/Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 10% dimethyl sulfoxide (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany) until use.

Fresh primary gastric tumor and nontumor gastric mucosa tissue samples from GC patients were transported to the laboratory in Hank's balanced salt solution medium (Gibco/Thermo Fisher Scientific) supplemented with 100 U mL^{-1} penicillin/streptomycin (GE Healthcare). Tissues were cut into small pieces using sterile scalpel blades and minced in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Corning) supplemented with penicillin/streptomycin and 3% FBS with the help of syringe needles and plungers. To obtain single-cell suspensions, the resultant dissociated tissues were passed through 70-μm cell strainers (BD Biosciences, San Jose, CA, USA) to remove tissue fragments. Cells were then centrifuged at 300*g* for 10 min at 4°C, and the pellet was incubated with erythrocyte lysis buffer for 10 min at room temperature. Cells were washed with supplemented RPMI-1640 medium, centrifuged and counted. Cell viability was assessed by an exclusion method using Trypan blue (Sigma-Aldrich) staining. Cell suspensions were then used for flow cytometric analysis, as well as for conventional and real-time PCR.

Tumor cell lines

K562 human erythroleukemia cells were gently donated by Dr Mercedes López (School of Medicine, University of Chile,

Santiago, Chile) and maintained in RPMI-1640 medium supplemented with penicillin/streptomycin and 10% FBS at 37°C and 5% CO_2. HeLa cell line transformed with CD1d or Mock insert was kindly provided by Dr Derek Doherty (Trinity College Dublin, Dublin, Ireland) and cultured in the same conditions.

Flow cytometry

Thawed PBMC were washed two times with PBS with 2% FBS. Approximately 200 000 PBMCs were stained in a V-bottom 96-well plate (Thermo Fisher Scientific) for 20 min at 4°C in the dark with a 1:1000 dilution of the fixable viability dye eFluor 780 (Affymetrix, Inc./Thermo Fisher Scientific). PBMCs were washed and stained for 30 min at 4°C in the dark with the following mAbs conjugated to fluorophores: anti-TCR Vα24-Jα18 fluorescein isothiocyanate (FITC; clone 6B11; BioLegend, San Diego, CA, USA), anti-TCR Vβ11 phycoerythrin (PE; Beckman Coulter, Inc., Brea, CA, USA), anti-CD3 PerCP-Cy5.5 (BioLegend), anti-CD4 V450 (BD Biosciences), anti-CD8a PE-Cy7 (BioLegend) and anti-NKG2D APC (BD Biosciences). Specificity of anti-TCR Vα24-Jα18 FITC mAb was tested with an FITC mouse immunoglobulin G1 (IgG1), κ isotype control (BioLegend), whereas an APC mouse IgG1, κ isotype control (Affymetrix) was used to test anti-NKG2D APC mAb specificity. Cells were analyzed in a BD LSRFortessa X-20 flow cytometer (BD Biosciences).

To analyze NKG2DL levels on K562 cell line, cells were incubated for 1 h at 4°C with purified mouse antihuman HLA-A/B/C PE (eBioscience, San Diego, CA, USA), MICA, MICB, ULBP-1, ULBP-2/5/6 (this mAb detects these three ligands, as human ULBP-2 shares 92% and 95% amino acid sequence identity with ULBP-5 and ULBP-6, respectively), ULBP-3 or ULBP-4 IgG2b mAbs (all from R&D Systems, Inc., Minneapolis, MN, USA). Purified mouse IgG2b (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as the isotype control. Cells were washed in PBS with 2% FBS and incubated for 30 min at 4°C in the dark with a goat antimouse IgG antibody conjugated to FITC (KPL/Sera Care Life Sciences, Milford, MA, USA). CD1d expression was also assessed on K562 cells by flow cytometry using an anti-CD1d PE mAb and its corresponding isotype control (both from Affymetrix). Cells were analyzed in a BD FACSCalibur flow cytometer (BD Biosciences).

Cells derived from the gastric tumor and mucosa were resuspended with 1% paraformaldehyde in PBS with 1% FBS and stained for MICA, MICB, ULBP-1, ULBP-2/5/6 and ULBP-3 overnight at 4°C in the dark, followed by incubation with a goat antimouse IgG antibody conjugated to FITC. Purified mouse IgG2b was used as the isotype control. In parallel, gastric tissue cells were incubated for 30 min at 4°C in the dark with combinations of the following mAbs: anti-EpCAM FITC (BioLegend), anti-CD1d PE and anti-CD19 PE-Cy7 (both from Affymetrix). Cells were fixed in 2% paraformaldehyde before flow cytometry, which was performed with a BD FACSCalibur flow cytometer. All flow cytometry data were analyzed using FlowJo version X software (Tree Star Inc., Ashland, OR, USA).

RNA extraction, reverse-transcription, conventional PCR and real-time PCR

After surgical resection, fresh gastric tumor and mucosa samples were immediately immersed in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and stored at −20°C until RNA extraction. Gastric tissue samples, K562 and HeLa cell lines were washed with PBS and resuspended at 1 million cells mL^{-1} of the same buffer. RNA was extracted with TRIzol (Life Technologies/Thermo Fisher Scientific), according to instructions of the manufacturer, and resuspended in 20 μL of RNAse-free water (HyClone/GE Healthcare). RNA concentration was measured with a Take3 Micro-Volume Plate (BioTek, Winooski, VT, USA) in a Synergy HT Multi-Mode Microplate Reader (BioTek). Samples were then treated with DNAse I (Thermo Fisher Scientific) and stored at −80°C. Complementary DNA was generated using an AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies, Santa Clara, CA, USA), according to instructions of the manufacturer. Complementary DNA quality was checked by conventional PCR against the housekeeping gene *β-actin* using the GoTaq Green Master Mix Kit (Promega, Madison, WI, USA). The relative expression of the *cd1d* gene against the housekeeping gene *hprt* was assessed by real-time PCR using Brilliant III SYBR Master Mix Kit in a Stratagene Mx3000P Real-Time PCR System (both from Agilent Technologies). The following program was used: 95°C for 15 s, 60°C for 15 s and 72°C for 15 s at 40 cycles. All real-time PCRs were performed in triplicates and raw data were analyzed using the MxPro software (Agilent Technologies). Expression values were calculated using the ΔΔCt method and expressed as the fold change relative to control samples. Primers used for PCRs are listed in Supplementary table 1.

iNKT cell expansion and cell sorting

PBMCs from GC patients and HDs were thawed, washed and resuspended in iNKT cell medium containing RPMI-1640 supplemented with 10% FBS, 100 U mL^{-1} penicillin/streptomycin, 0.25 μg mL^{-1} amphotericin B (Thermo Fisher Scientific), 0.05 mM L-glutamine, 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 100 μM 2-β-mercaptoethanol, 1 mM sodium pyruvate, 1% of essential amino acids, 1% of non-essential amino acids (all from Thermo Fisher Scientific), 200 U mL^{-1} of IL-2 and 200 U mL^{-1} of IL-7 (both from Miltenyi Biotec, Bergisch Gladbach, Germany) and 100 ng mL^{-1} of α-GalCer (Enzo Life Sciences, Inc., Farmingdale, NY, USA). Cells were cultured for 30 days in a U-bottom 96-well plate (Thermo Fisher Scientific) with fresh iNKT cell-medium replacement every 5 days without α-GalCer. Specific iNKT cell expansion was inspected by flow cytometry every 10 days. After 30 days in culture, expanded iNKT cells were harvested and stained with anti-CD3 PerCP-Cy5.5, anti-TCR Vα24-Jα18 FITC and anti-TCR Vβ11 PE for 30 min at 4°C in the dark. Cells were resuspended in 500 μL of FBS and incubated for 2 min with 7-aminoactinomycin D (eBioscience) for cell sorting in a BD FACSAria III cell sorter (BD Biosciences). Upon expansion,

iNKT cells corresponded to approximately 20% of total cells in culture, and an average of 96% iNKT cell purity was obtained after cell sorting.

Cytokine production and CD107a expression

Purified iNKT cells from GC patients and HD were seeded in a U-bottom 96-well plate in the presence of 3 μL of anti-CD107a APC-Cy7 (BioLegend) and stimulated with 10 ng mL^{-1} of PMA plus 50 ng mL^{-1} of ionomycin (both from Sigma-Aldrich) and incubated for 1 h at 37°C and 5% CO_2, followed by addition of 2 μM of monensin (GolgiPlug, BD Biosciences) and 1 μg mL^{-1} of brefeldin A (GolgiStop, BD Biosciences). Cells were then incubated for 5 h, harvested and stained with anti-CD3 APC (BioLegend), anti-TCR Vα24-Jα18 FITC and anti-TCR Vβ11 PE. Cells were permeabilized for 20 min with Cytofix/Cytoperm Kit (BD Biosciences), and intracellular staining was performed using, separately, anti-IL-2 PE-Cy7 (clone MQ1-17H12) (eBioscience), anti-IFNγ PE-Cy7 (clone B27), anti-TGF-β PE-Cy7 (clone TW4-2F8) or anti-IL-10 PE-Cy7 (clone DES3-9D7; all from BioLegend). Finally, cells were resuspended in FACS buffer and analyzed in a BD LSRFortessa X-20 flow cytometer.

iNKT cells and tumor cells coculture and CD107a degranulation assays

Purified iNKT cells from GC patients and HDs were cocultured with K562 cells in RPMI-1640 medium supplemented with penicillin/streptomycin and 10% FBS with different effector-to-target cell ratios (1:1, 2:1 and 10:1) in U-bottom 96-well plates. iNKT cells cultured alone and iNKT cells stimulated with 10 ng mL^{-1} of PMA plus 50 ng mL^{-1} of ionomycin were used as negative and positive controls, respectively. Each condition was tested in triplicates; 3 μL of anti-CD107a APC-Cy7 was added to each well in the beginning of cocultures, and plates were centrifuged at 30*g* for 1 min to ensure cell-to-cell contact. Cocultured cells were then incubated for 1 h at 37°C and 5% CO_2, followed by addition of 3 μM of monensin and 1 μg mL^{-1} of brefeldin A. Cells were further incubated for 5 h, harvested and stained with anti-CD3 PerCP-Cy5.5, anti-TCR Vα24-Jα18 FITC, anti-TCR Vβ11 PE and anti-NKG2D APC mAbs. Next, cells were permeabilized for 20 min with Cytofix/Cytoperm Kit and stained intracellularly with antihuman IFNγ PE-Cy7. Finally, cells were resuspended in FACS buffer and analyzed in a BD LSRFortessa X-20 flow cytometer.

Statistical analysis

Normality was tested by the Kolmogorov–Smirnov test and significance was determined by either a Mann–Whitney *U*-test, Wilcoxon matched-pairs signed-rank test, Student's *t*-test or multiple *t*-test, with a confidence interval of 95%. Patient survival was analyzed by the Kaplan–Meier method, and the log-rank test was performed to assess survival differences. *P*-values lower than 0.05 were considered statistically significant. For all analysis, GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) was employed.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.