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Characterization of immune responses in gastric cancer patients: A possible impact of *H. pylori* to polarize a tumor-specific type 1 response?

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Abstract Recently, we were able to show that *Helicobacter pylori*-positive gastric cancer (GC) patients have a significantly better survival after the complete resection of their tumor compared to *H. pylori*-negative GC patients. *H. pylori* is known to polarize an immune response towards a type 1 cytokine profile and tumor-specific type 1 cytokine responses are associated with protection from tumor challenge and T-cell-mediated tumor regression. Therefore, we hypothesized that the improved survival in *H. pylori*-positive patients may be secondary to the induction of a GC-specific type 1 T cell response. To characterize the anti-tumor immune response in GC patients we analyzed tumor-infiltrating lymphocytes (TIL) isolated from primary tumors. The CD3+ T cell population contained 50% CD4+ (range 0.4—81%) and 39% CD8+ cells (range 22—53%). The number of B cells (CD19+, $P = 0.03$) was significantly increased and the number of T cells (CD3+, $P = 0.02$) significantly decreased in intestinal compared to diffuse type of tumors. Four tumor cell lines were established from primary GCs and three from lymph node metastases. T cell cultures were established from isolated TIL from four *H. pylori*-positive and one *H. pylori*-negative GC patients and tested for tumor-specific cytokine secretion. Eight of ten T cell cultures derived from *H. pylori*-

Abbreviations: TIL, tumor-infiltrating lymphocytes; GC, gastric carcinoma; *H. pylori*, *Helicobacter pylori*.

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positive patients secreted both IFN-γ and IL-5 after restimulation with autologous tumor cells. The only tumor-specific TIL line expressing a dominant IL-5 response was derived from an *H. pylori*-negative patient.

Introduction

Gastric carcinoma (GC) is one of the most frequent cancers in industrialized countries with more than 192,000 new cases in Europe every year [1]. Despite improved surgical and chemotherapeutic treatment modalities, the prognosis is poor, and treatment for gastric cancer has not much improved in the last decades [1—3]. More than 50% of the patients treated curatively for gastric cancer will develop a recurrence within 5 years.

The immune system plays an important role in controlling tumor outgrowth and progression [4]. The poor prognosis of patients with gastric cancer suggests that gastric tumor-specific T cells are either not sufficiently induced or suppressed by the induction of tolerance. Different forms of tolerance can be induced by tumors [5]. Immune deviation is one possible form of tolerance and is defined as the induction of a non-therapeutic immune response [6]. Immune responses can be segregated into type 1 or type 2 responses based on the cytokines secreted by antigen-specific CD4 and CD8 effector T cells [7,8]. Type 1 T cells (Th1/Tc1) characteristically secrete IFN-γ and/or TNF-α and induce cellular immune responses, whereas type 2 Tcells (Th2/Tc2) secrete IL-4, IL-5, and IL-10 and promote humoral immune responses. Recently, it has been shown that the therapeutic efficacy of tumor-specific Tcells depends on the generation of a type 1 (IFN-γ) response [6,9—12]. In contrast, the induction of tumor-specific type 2-polarized responses (IL-4 or IL-5) correlates with tumor progression in patients with melanoma and renal cancer [13].

Although tumor-infiltrating lymphocytes (TIL) have been detected in gastric tumors, little is known about their specificity and function [14,15]. Previous analyses of lymphocyte subpopulations in gastric cancer patients did not examine tumor-specific immune responses [16—18]. Here, we characterize the phenotype of TIL from gastric cancer specimens and investigate their tumor-specific cytokine release upon restimulation with autologous tumor.

Materials and methods

Patients

Fifty-one consecutive patients with gastric adenocarcinoma, who underwent surgery between 2002 and 2004, at the Department of Surgery, Klinikum Grosshadern, LMU, Munich, Germany, and of whom tumor specimens were available for culturing were enrolled in this study. Each patient provided written informed consent. The study protocol was approved by the ethics committee of the Klinikum Grosshadern (No. 240/01). Subtotal or total gastrectomy in combination with N2 lymph node dissection was performed in all patients. The resected tumors were classified by depth of invasion (pT), lymph node metastases (pN), and histological type according to the Laurén classification at the Department of Pathology, LMU Munich, Germany. The Laurén classification differentiates between diffuse and intestinal type of tumors. The diffuse type is characterized by separated single cells or small clusters of cells that diffusely infiltrate the layers of the gastric wall. Intestinal type carcinomas are distinguished by a glandular structure, resembling cells that are present in intestinal neoplasm. Carcinomas that do not fit into one of these types but show overlapping characteristics are classified as mixed tumors.

Tissue sampling

Biopsy specimens were obtained from tumor tissue and from macroscopically tumor-free antrum and corpus mucosa. Corpus and antrum specimens were placed in 3.7% neutral formalin for paraffin-embedding and histological examination of *Helicobacter pylori* infection (Warthin-Starry stain by M. Stolte, Bayreuth). Biopsies for cell isolation were transported in sterile RPMI-1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 2× gentamicin (100 μg/ml, Cambrex Bio Science) on ice. The tumor tissue was minced with a surgical scalpel in pieces of 2 × 2 mm and digested in a triple enzyme solution containing 0.02 mg/ml deoxyribonuclease I, 0.25 mg/ml collagenase and 0.1 mg/ml hyaluronidase (Sigma, Steinheim, Germany) overnight at room temperature.

Culturing of T cells and tumor cells

Digested tumor specimens were filtered through a 100-μm nylon mesh (BD Biosciences, Erembodegem, Belgium). Cells were washed and cultured either in tissue culture flasks with complete medium (CM, RPMI-1640 supplemented with 10% FBS, Invitrogen, Karlsruhe, Germany; 0.2 mM NEAA, 2 mM sodium pyruvate, 4 mM L-glutamine, and 50 μg/ml gentamicin, Cambrex Bio Science) to establish tumor cell lines or in multiple wells of a 24-well plate in X-Vivo-15 medium (Cambrex Bio Science) supplemented with 2400 IU IL-2/ml (Proleukin, Chiron, Ratingen, Germany) to generate tumor-infiltrating T cell (TIL) cultures (also referred to as T cell cloids because they were initiated from a limited sample of TIL). When possible, T cell cloids were cryopreserved in liquid nitrogen and subsequently thawed for use in the cytokine release assays.

Characterization of tumor-infiltrating T cells by flow cytometry

Freshly digested samples were washed in FACS buffer (0.5% BSA, 0.1% sodium azide in phosphate-buffered saline (PBS) and incubated with 10% autologous serum in PBS to block Fc receptors. Four-color staining was performed using the following monoclonal antibodies and isotype controls: CD3-PE, CD62L-PE, CCR7-PE, IgG1-PE, CD8-FITC, CD19-FITC,

IgG1-FITC, CD4-PerCP-Cy5.5, CD45-PerCP-Cy5.5, IgG1-PerCP-Cy5.5, CD56-APC, CD25-APC, CD69-APC, CD45RA-APC, CD45RO-APC, IgG1-APC (BD Biosciences). The cells were stained for 30 min at 4°C, washed in FACS buffer, and fixed in 1% paraformaldehyde in PBS. Acquisition of data was performed on a FACS-Calibur (BD Biosciences), and collected data were analyzed using WINMDI28 software (URL: http://facs.scripps.edu).

Proliferating T cells in the 24-well plates were expanded in culture flasks in X-Vivo-15 medium supplemented with IL-2 (2400 IU/ml) at a starting density of 0.5×10^6 cells/ml. The proportion of CD4 and CD8 T cells was determined by flow cytometry using CD3, CD8, and CD4 antibodies as described above.

Characterization of tumor cells

Primary tumor cell cultures were fed weekly with fresh CM. Passaging of tumor cells was performed using 1 mM EDTA in PBS, if necessary followed by a short trypsinization. MHC expression on tumor cells was determined using HLA-A, B, C-FITC and HLA-DR-PE (Clone G46-2.6 and L243, respectively, BD Biosciences), and analyzed by flow cytometry as described above.

For cytospins, tumor cells were washed twice in PBS, cyto-centrifuged on microscope slides (5×10^4 cells per spot, Shandon Cytospin 2), air dried, fixed with acetone, and stained for epithelial, fibroblast, and lymphoid surface markers for 1 h at room temperature. Primary antibodies were KL-1 (detecting a large spectrum of cytokeratines, 0.3 ng/ml, Immunotech, Marseille, France), Ber-Ep4 (EpCAM, 5 μg/ml, Dako, Glostrup, Denmark), alpha-smooth muscle actin (0.7 μg/ml, Dako), Vim 3B4 (vimentin, 0.6 μg/ml, Dako), T200 (CD45, 4.5 μg/ml (Dako), CD31 (2 μg/ml, Immunotech), CD68 (Serotec, 5 μg/ml), CD209 (1.25 μg/ml, BD Biosciences), and the isotype controls MOPC-21 (IgG1, 10 μg/ml, Sigma) and UPC-10 (IgG2a, 2 μg/ml, Sigma, St. Louis, USA). Detection was performed as described before [19]. The slides were washed in PBS pH 7.4, and a peroxidase-conjugated rabbit anti-mouse IgG antibody (1:200, Dako) was added for 30 min. The peroxidase reaction was developed in 3-amino-9-ethylcarbazole (0.25 mg/ml in 0.1 M sodium acetate buffer, pH 4.9) containing 0.003% H_2O_2 for 10 min. The slides were counterstained with Mayer's hemalun solution and mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Cytokine release assay

Cytokine release assays were performed as described elsewhere [20] with the following modifications: T cells (1×10^6 cells) were washed and cultured alone or stimulated with tumor cell lines (0.2×10^6 cells), frozen autologous tumor samples, or immobilized anti-CD3 (OKT-3) in 1 ml of X-Vivo-15 supplemented with 60 IU IL-2/ml in a 48-well tissue culture plate at 37°C, 5% CO_2 for 20 h. The tumor targets included autologous tumor cells either as cell lines or as fresh frozen samples (GC24, GC30). The renal cancer cell lines RCC26 and RCC53 (kindly provided by Dr. D.J. Schendel, Munich, Germany) and the melanoma cell lines MEL118 and MEL119 (established at the Earle A. Chiles Research Institute, Portland, OR) were used as controls. Supernatants were analyzed for the presence of the cytokines IFN-γ and IL-5 in duplicate measurements by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany). T cell cloids were categorized according to their IFN-γ and IL-5 cytokine secretion upon stimulation with autologous tumor. Predominant tumor-specific release of IFN-γ with little or no secretion of IL-5 was considered a type 1 response, predominant tumor-specific release of IL-5 with little or no secretion of IFN-γ was considered a type 2 response. Tumor-specific expression of both cytokines was considered a mixed type of response. Tumor cell lines cultured alone did not secrete any IFN-γ or IL-5.

Statistical analysis

Differences between the means of groups were analyzed with the SPSS software (SPSS GmbH Software, Munich, Germany) using the Mann—Whitney test or, where indicated, the Kruskal—Wallis test. Two tailed *P* values below 0.05 were considered significant.

Results

Patient demography and tumor staging

From January 2002 until July 2005, 51 gastric cancer patients (33 male, 18 female, age range 40—91) were enrolled into the study. Twenty-one patients (41%) had gastric carcinomas of the intestinal type and 30 patients (59%) had mixed or diffuse type tumors. Most tumors were localized in the upper part of the stomach (corpus/cardia) or spread over the whole stomach. Tumor cultures were initiated from primary stomach carcinomas from all 51 patients. From 16 patients, additional cultures were started from local lymph nodes (LN), which were suspected to contain tumor metastases. The establishment of gastric carcinoma cell lines was difficult. However, long-term tumor cell lines with over 20 passages were successfully established from 3 primary tumors (GC9 Sto-Tu, GC34 Sto-Tu, and GC50 Sto-Tu) and 3 LN metastases (GC1 LN-Tu, GC9 LN-Tu, and GC20 LN-Tu). A short-term primary culture (less than 5 passages) was established from tumor tissue of patient GC7 (GC7 Sto-Tu). Table 1 summarizes the pathological features of the tumors from 27 patients whose isolated cells were further analyzed in this study. All established tumor cell lines originated from tumors of the corpus/cardia and from patients with advanced gastric cancer (stage III or IV, Table 1).

Phenotypic analyses of freshly isolated tumor-infiltrating lymphocytes

Analysis of the tumor-infiltrating lymphocytes (TIL) revealed variable numbers of lymphocytes in the tumor tissues of individual patients. Cells in the lymphocyte gate, which were 85%—100% CD45+, ranged from 11% to 94% of the total cell count (not shown). Phenotypic analysis of the CD45+ cells revealed a T cell fraction ranging from 48% to 88% and a B cell fraction ranging from 5% to 34%. A significant percentage of recently activated T cells (CD3+CD69+) were

Table 1 Patient demography and tumor staging

	m/f	Age (Year)	Tumor type (Laurén)	G	Localization	T	N		M		UICC Stage	H.p.
GC1	m	74	Mixed	G3	Whole stomach	pT2	pN2	10/14[a]	M1	L[b]	IV	pos
GC7	m	79	Diffuse	G3	Corpus/cardia	pT2	pN1	6/33	M0		II	pos
GC9	m	72	Intestinal	G3	Cardia	pT2(b)	pN3	20/38	M0		IV	neg
GC20	f	63	Intestinal	G3	Corpus	pT3	pN2	15/44	M0		III	pos
GC21	f	70	Intestinal	G2-3	Cardia	pT2b	pN1	4/34	M1	P	IV	pos
GC24	m	75	Diffuse	G3	Corpus	pT2b	pN1	3/28	M0		II	pos
GC26	m	60	Diffuse	G3	Antrum	pT2b	pN3	23/25	M0		IV	pos
GC28	m	79	Mixed	G3	Whole stomach	pT2(b)	pN2	8/13	M0		III	pos
GC29	m	74	Intestinal	G2	Cardia	pT2(b)	pN1	1/21	M0		II	pos
GC30	m	83	Mixed	G3	Corpus	pT2(b)	pN3	16/21	M0		IV	pos
GC31	f	66	Intestinal	G3	Corpus	pT2(b)	pN2	11/31	M0		III	pos
GC32	m	60	Diffuse	G3	Whole stomach	pT2(b)	pN3	30/31	M0		IV	pos
GC34	m	49	Diffuse	G2	Cardia	pT2b	pN1	5/16	M1	L,P	IV	neg
GC37	m	58	Mixed	G3	Whole stomach	pT2b	pN3	20/25	M0		IV	pos
GC38	f	50	Intestinal	G3	Corpus/cardia	pT3	pN2	12/20	M1	Pe	IV	neg
GC46	f	76	Diffuse	G3	Antrum	pT3	pN3	16/17	M1	Lo	IV	pos
GC50	m	66	Intestinal	G2	Cardia	pT2b	pN3	18/39	M0		IV	pos
GC51	m	57	Intestinal	G3	Corpus	pT2b	pN0	0/16	M0		I	pos
GC52	m	66	Mixed	G3	Corpus/cardia	pT2b	pN2	7/45	M0		III	pos
GC54	m	58	Diffuse	G3	Whole stomach	pT4	pN3	19/19	M1	L	IV	pos
GC60	f	86	Intestinal	G3	Antrum	pT3	pN1	3/5	M0		III	neg
GC62	f	63	Intestinal	G3	Whole stomach	pT2b	pN2	7/28	M0		III	pos
GC63	f	62	Diffuse	G3	Corpus	pT3	pN2	12/18	M0		III	pos
GC64	m	79	Diffuse	G3	Cardia	pT2b	pN1	6/23	M0		II	neg
GC67	f	35	Mixed	G3	Cardia	pT2b	pN2	13/22	M1	O	IV	pos
GC68	m	65	Intestinal	G3	Corpus	pT2b	pN2	9/26	M0		III	pos
GC70	m	38	Intestinal	G3	Corpus	pT3	pN1	5/17	M0		III	pos

[a] Number of LN with metastases/total number of LN.
[b] Localization of distant metastases L: liver; P: lung, Pe: peritoneum, Lo: local, or O: ovaries.

detected in the tumors of two gastric cancer patients (Table 2). The fraction of activated B cells (CD19+CD69+) remained below 3% in all analyzed samples. Comparison of T and B cell fractions in the context of tumor localization or tumor stage did not reveal any significant correlation. However, T cell infiltration was significantly increased in diffuse or mixed type tumors compared to intestinal carcinomas ($P = 0.02$). In contrast, the fraction of infiltrating B cells was significantly increased in intestinal type of tumors ($P = 0.03$, Fig. 1).

Table 2 Lymphocytes in freshly digested gastric tumor tissue

Percent of CD45	CD3+	CD3+CD69+	CD19+	CD19+CD69+
GC38 Sto-Tu	48	1.2	34	0
GC46 Sto-Tu	82	0	5	0
GC51 Sto-Tu	70	0.3	22	0.1
GC52 Sto-Tu	78	0.1	20	0
GC62 Sto-Tu	63	0.7	27	0.3
GC63 Sto-Tu	86	31	7	2.7
GC64 Sto-Tu	88	53	7	2
GC67 Sto-Tu	80	0.6	16	0
GC68 Sto-Tu	71	0.8	24	0.2
GC70 Sto-Tu	80	1	9	0.2

Since the lymphocytes had not been separated from any tumor cells or other accompanying cells out of the digested tissues, T cells were further analyzed by setting a lymphocyte gate (FSC-SSC) as well as a gate for the CD3+ cells (CD3-SSC). The number of CD3+ T cells varied largely in the different TIL samples and ranged from 1.3% to 69% (mean 26.7%) of the totally acquired cells (Table 3). To compare T cell subsets, the expression of T cell markers was analyzed as a percentage of CD3+ cells. The frequency of CD8+ and CD4+ T cells did not significantly differ when comparing infiltrating cells in intestinal and diffuse gastric cancers (not shown). The CD3+ cells consisted of equal or higher numbers of CD4+ cells (mean 50%) as compared to CD8+ T cells (mean 39%) except for two patients with antral tumors (GC46 and GC60), who had lower numbers of infiltrating CD4+ T cells in their tumors as compared to tumors located in the upper part of the stomach or in the whole stomach ($P = 0.09$, Table 3, Fig. 2). This was also reflected in the amount of CD4+CD62L− T cells, which tended to be higher in tumors located in the upper part or the whole stomach compared to tumors in the antrum (Table 3 and not shown). No notable changes in the frequency of infiltrating CD4+ T cells were observed between the different tumor types as classified by Laurén (not shown) or tumor stage (Fig. 2). In contrast, the CD8+ T cell fraction tended to decrease with increasing tumor stage ($P = 0.05$, Fig. 2). We did not observe differences in frequency of CD3+ T cells expressing the homing receptor CD62 L or chemokine

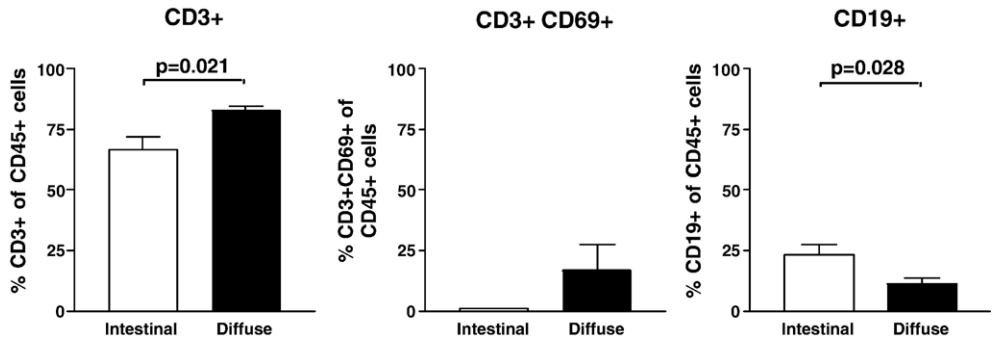

Figure 1 Infiltration of T and B lymphocytes in gastric carcinoma. Freshly isolated cells from tumor tissue of gastric cancer patients were analyzed for CD45, CD3, CD19 and CD69 expression by flow cytometry. Analyzed tumor specimens were sorted according to the Laurén classification in diffuse (n = 5) or intestinal types (n = 5). Values are depicted as the percentage of CD45+ cells. Mean values and SEM are indicated for each group.

receptor 7 (CCR7), or in frequency of naïve (CD45RA+) T cells when comparing tumor type or location (Fig. 3). Most CD3+ T cells expressed CD45RO, which is a marker for a memory phenotype (not shown). The number of natural killer (NK) T cells (CD3+ CD8+ CD56+) or NK cells (CD3− CD8+CD56+) was negligible (less than 3%, not shown).

Characterization of established gastric tumor and T cell cloids

Established tumor cell lines were characterized using a panel of cell-type-specific markers. Primary gastric cancer cell lines revealed high expression of cytokeratines and the epithelial cell adhesion molecule (EpCAM) demonstrating their origin from simple epithelia (Table 4). Interestingly, most of the cell lines originated from lymph node metastases strongly expressed vimentin. All tumor cell lines showed a low expression level of HLA-A, B, C, but no expression of HLA-DR (data not shown).

The expansion of T cells from tumor or lymph node tissue in 24-well plates resulted in several independent TIL cultures or lymph node lymphocyte cultures (LN T cells) from a single patient. From initial cell cultures obtained from tumor tissues of 51 patients, 61% (31 tissues) resulted in one or more wells with sufficient cell growth to perform at least three passages of the TIL. About 40% of the cultured lymph node cells (11 of 26 lymph nodes) led to sufficient growth of LN T cells. These T cell cloids showed phenotypic heterogeneity of CD4 and CD8 T cells. In 18 of 32 characterized cloids the majority of the cells were CD4+ and in 9 of 32 cloids more CD8+ cells were found (not shown). Most of the cytokine release assays were performed with cloids containing predominantly CD8+ T cells.

Polarization of TIL and LN T cells after polyclonal and tumor-specific activation

Fig. 4 illustrates the IFN-γ and IL-5 cytokine release from TIL and LN T cell cloids after polyclonal stimulation with anti-CD3. Almost all cloids secreted high levels of IFN-γ after polyclonal stimulation. Four cloids showed similar levels of IFN-γ and IL-5 cytokine expression. Hence, the majority of the cloids were polarized to a type 1 cytokine profile.

To examine whether T cell cloids contained tumor-specific T cells, cytokine responses were analyzed after

Table 3 Characterization of infiltrating T cells in gastric tumor tissue

Percent of CD3	Total CD3+	CD8+	CD8+ CD62L+	CD8+ CD62L−	CD4+	CD4+ CD62L+	CD4+ CD62L−
GC21 Sto-Tu	1.3	22	n.d.	n.d.	81	n.d.	n.d.
GC29 Sto-Tu	7	53	n.d.	n.d.	51	n.d.	n.d.
GC31 Sto-Tu	8	49	n.d.	n.d.	44	n.d.	n.d.
GC37 Sto-Tu	6	29	n.d.	n.d.	60	n.d.	n.d.
GC38 Sto-Tu	19	36	n.d.	n.d.	58	n.d.	n.d.
GC46 Sto-Tu	10	34	0	40	0.4	0	10
GC51 Sto-Tu	47	35	2	34	61	14	48
GC52 Sto-Tu	69	24	5.4	21	69	34	36
GC54 Sto-Tu	32	36	2.1	34	58	11	49
GC60 Sto-Tu	15	50	0.8	49	15	3	11
GC62 Sto-Tu	22	44	0.3	44	40	4	39
GC63 Sto-Tu	13	46	1.3	43	55	7	46
GC64 Sto-Tu	22	42	n.d.	n.d.	50	n.d.	n.d.
GC67 Sto-Tu	21	42	n.d.	n.d.	47	2.3	48
GC68 Sto-Tu	20	38	1	38	48	7	52
GC70 Sto-Tu	15	39	2	37	61	11	49

n.d., not determined.

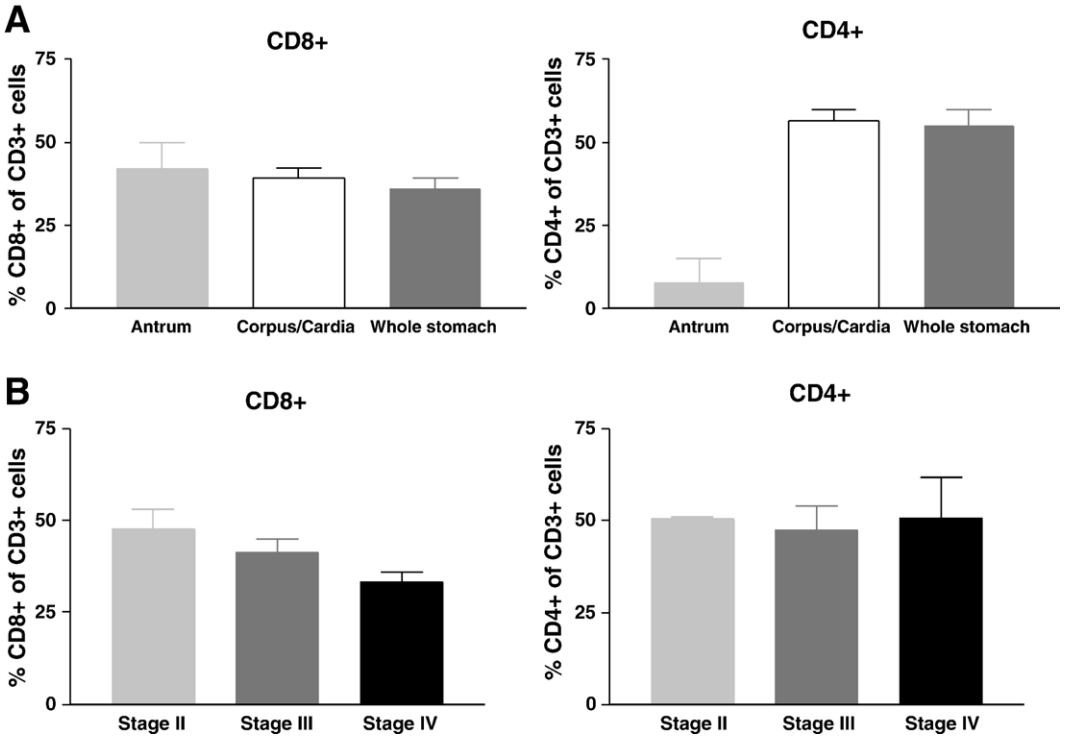

Figure 2 Evaluation of CD8 and CD4 T cell infiltration in gastric carcinomas. Tumor specimens were analyzed according to (A) the tumor localization in the stomach (antrum, $n = 2$; corpus/cardia, $n = 10$; whole stomach, $n = 4$) or (B) the tumor stage as defined by UICC (stage II, $n = 2$; stage III, $n = 7$; stage IV, $n = 6$). Values are depicted as the percentage of CD3+ cells. Mean values and SEM are indicated for each group. Groups were compared using the Kruskal—Wallis test.

stimulation with autologous tumor cells derived from the established cell lines or from freshly frozen digested tumor tissue. The tumor cell lines had been passaged 2 to 5 times at the time point of analysis. Tumor-specific IFN-γ and IL-5 cytokine release were determined as the markers for type 1 and type 2 responses, respectively. If the same cloid, in response to stimulation with specific tumor, produced both cytokines, the response was judged to be a mixed response. Fig. 5 shows tumor-specific cytokine responses for ten different cloids of five different patients. T cells from cloid GC9 TIL3 responded strongly to stimulation with autologous tumor, secreting 3- to 4-fold increased IL-5 levels as compared to GC9 TIL3 cells cultured alone (medium) or with two unrelated tumor cell lines RCC53 and RCC26. Since the same cloid secreted only low levels of IFN-γ, this cloid clearly displayed a type 2 response (Fig. 5). Cloid GC24 LN6 secreted high levels of IFN-γ and low levels of IL-5 upon stimulation with freshly digested autologous tumor, displaying a type 1 response (Fig. 5). The other TIL or LN cell cloids secreted both tumor-specific IFN-γ and IL-5 (Fig. 5). While sufficient numbers of cells were not available in all cases, we could show for one patient that secretion of cytokines by T cells stimulated with freshly digested tumor was completely blocked by anti-class I antibody (not shown). Not all CRAs with autologous cells displayed a tumor-specific response. None of the LN cloids isolated from patient GC1 released cytokines in a tumor-specific manner when incubated with autologous GC1 LN-Tu cells (not shown). To summarize, eight of ten TIL cloids displayed mixed tumor-specific cytokine responses by secreting both IFN-γ and IL-5. One cloid clearly displayed a type 1 response and one cloid was type 2 polarized.

To further differentiate between the mixed responses, we determined the IFN-γ to IL-5 ratios of the tumor-specific responses for each cloid. Tumor-specific responses with IFN-γ:IL-5 ratios higher than 2 were considered type 1 and ratios below 0.5 were regarded type 2. Tumor-specific responses with ratios between 0.5 and 2 were considered mixed type of responses. From the ten T cell cloids, two displayed a dominant type 2 response, three a mixed response and five a dominant type 1 response upon tumor-specific stimulation (Fig. 6). Thus, the mixed cytokine responses seemed to be more directed towards type 1 cytokine responses. This reflects the high tumor-specific IFN-γ expression in almost all T cell cloids. Interestingly, the two cloids with low IFN-γ secretion upon autologous stimulation (GC9 TIL3 and GC9 TIL4) appeared to be from a *H. pylori*-negative patient.

Discussion

The presence of tumor-infiltrating lymphocytes (TIL) is associated with a positive clinical outcome in patients with renal, ovarian, esophageal, and colorectal carcinomas [21]. The polarization of the TIL seems to be crucial for their therapeutic efficacy. Dudley et al. showed that especially type-1 polarized tumor-specific cells correlated with tumor regression in patients with melanoma [12]. The present study characterized the phenotype of gastric cancer-infiltrating lymphocytes as well as the functional activity of the

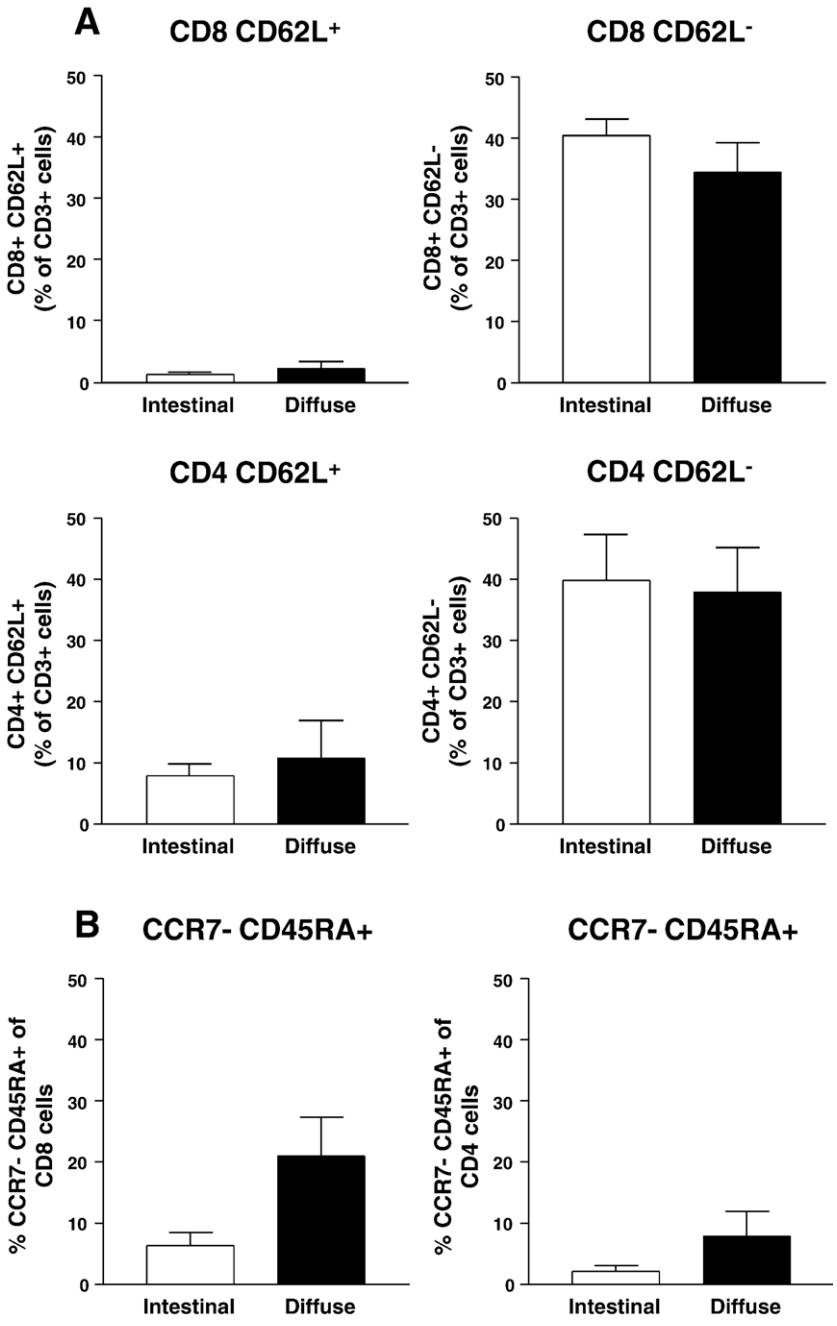

Figure 3 Characterization of tumor-infiltrating T lymphocytes in gastric carcinoma. Freshly isolated cells from tumor tissue of gastric cancer patients were analyzed for (A) CD8, CD4, CD3, and CD62L expression (n = 4 or 5 per group) or (B) CD8, CD4, CCR7, and CD45RA expression (intestinal: n = 4, diffuse: n = 6) by flow cytometry. Analyzed tumor specimens were sorted according to the Laurén classification in diffuse or intestinal types. Mean values and SEM are indicated for each group.

TIL lines derived from these tumors. T cells were more frequently detectable in gastric carcinomas compared to B cells. T cell and B cell proportions appeared to be significantly different in diffuse versus intestinal type of tumors. In contrast, no correlation with tumor localization, tumor stage or the tumor type according to the Laurén classification was detected within the T cell population for most other surface markers.

CCR7 and CD62L (L-selectin) mediate homing of T cells to secondary lymphoid organs via high endothelial venules (HEV) and therefore characterize naïve or central memory lymphocytes that perform routine immunosurveillance. The absence of CCR7 expression on most of the TIL in gastric cancer patients suggests that these cells have been activated, which is known to induce downregulation of CCR7 [22]. The expression of CD45RO on most of the infiltrating T cells found in gastric carcinomas likely points towards the presence of effector memory cells [23].

The activation marker CD69 has been described to be expressed on virtually all tissue-infiltrating lymphocytes in

Table 4 Immunohistochemical characterization of primary cell cultures of tissue from gastric cancer patients

Patient tumor origin[a]		GC1 LN	GC7 Sto	GC9 LN	GC9 Sto	GC20 LN	GC34 Sto	GC50 Sto
Cell type	Marker							
Epithelial cells	Cytokeratins	<10	>90	nt	20—40	—	>80	>80
	EpCAM	<5	80	>80	30—70	<5	nt	nt
Fibroblasts	Vimentin	85[b]	—	<5	>80	80	nt	nt
	Alpha-smooth muscle actin	nt	—	—	40	—	nt	nt
Leukocytes	CD45	—	nt	—	—	—	—	—
Macrophages	CD68	—	nt	<5	<5	—	—	—
Dendritic cells	CD209	nt	nt	—	—	—	—	—
Endothelial cells	CD31	—	—	—	—	—	—	—

nt: not tested; —: negative.
[a] LN: lymph node, Sto: stomach.
[b] Percentage of positive cells, <5: small number of positive cells detectable.

lymphoid and non-lymphoid tissue but not on lymphocytes in the peripheral blood [24,25]. In line with these reports, we did not detect CD69+ T cells in the peripheral blood of gastric cancer patients (not shown). However, we detected CD69+ TIL in only two out of ten gastric tumors. One explanation is that the tumor induced the activation of T cells in a minority of patients. However, this is inconsistent with the identification of tumor-specific T cells in the TIL of a majority of patients studied. Alternatively, the enzymatic digestion used during the isolation process may have affected the expression of CD69 [24].

NK cells are non-specific effector cells. The degree of tumor-infiltrating NK cells has been described to be positively correlated to a better prognosis in gastric cancer patients [26]. Our analysis did not reveal significant numbers of NK cells in the tumors of our patients. A possible explanation for this could be that flow cytometric analysis is less sensitive than immunohistochemistry, to detect NK cells in the tumors [27].

Previously, the CXC chemokine IL-8 was shown to be significantly higher expressed in diffuse gastric tumors than in intestinal tumors [28]. In addition, the chemokine Groα was only detected in diffuse carcinoma cells but not in

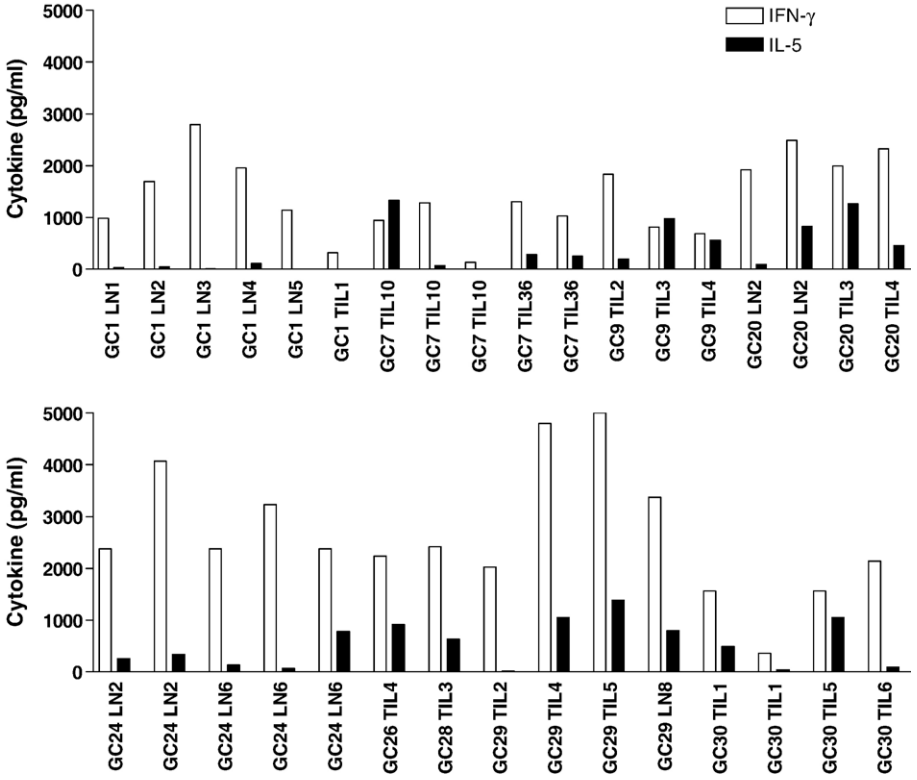

Figure 4 Polyclonal activation of T cell cultures derived from tumor or lymph node tissues of gastric cancer patients. After expansion in IL-2, T cells derived from tumor (TIL) or lymph node (LN) tissues were polyclonally activated with immobilized anti-CD3 (10 μg/ml). To examine the type of polarization (type 1 or 2), IFN-γ and IL-5 release was determined in the supernatant after 24 h by ELISA. Non-specific cytokine release of the medium control was subtracted.

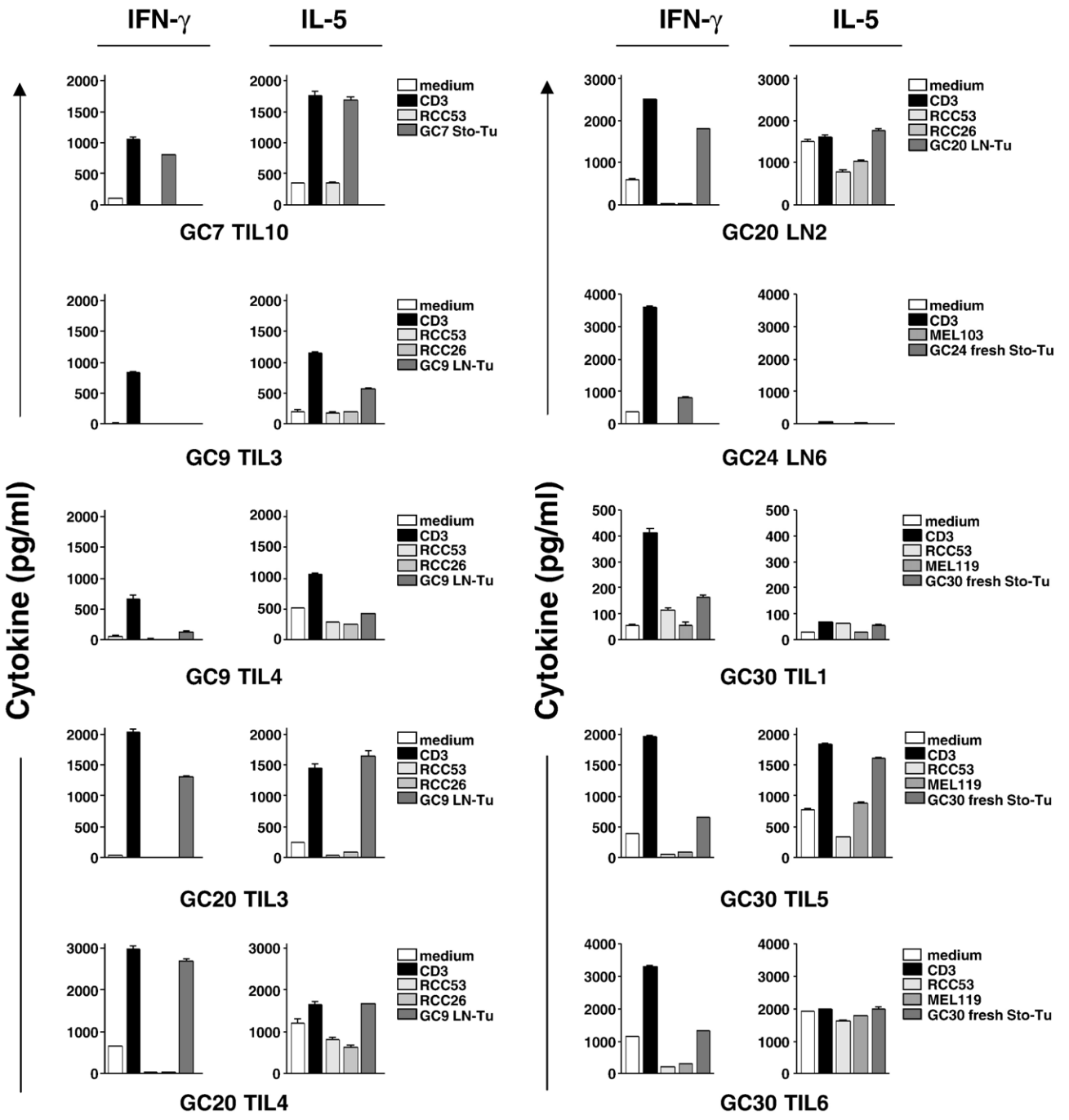

Figure 5 Tumor-specific cytokine responses in TIL or LN T cell cultures derived from gastric cancer patients show a mixed type 1/type 2 profile. TIL or LN T cell cloids were cultured with or without autologous or control tumor cell lines or freshly digested tumor. Stimulation with anti-CD3 served as a positive control. IFN-γ and IL-5 release were determined in the supernatants after 24 h. Means of duplicate measurements and SEM are indicated.

intestinal tumor cells. While these chemokines are known to be strong angiogenic factors, they also induce chemotactic activity in T cells. Therefore, an increased expression of chemokines may have contributed to the increased number of T cells that were detected in diffuse gastric carcinomas compared to intestinal type of tumors.

In addition to the characterization of tumor-infiltrating lymphocytes, this study aimed to determine the presence of tumor-specific T cells in primary tumor or lymph nodes from patients with gastric carcinoma and to analyze their tumor-specific cytokine response. The cytokine release assay (CRA) for the detection of tumor-specific T cells is a well-established and reproducible method. Chang et al. analyzed the tumor vaccine draining lymph nodes (TVDLN) of patients with advanced melanoma after vaccination of these patients with irradiated autologous tumor cells [29]. The isolated lymphocytes from these TVDLN secreted GM-CSF and IFN-γ upon stimulation with autologous but not allogeneic tumor.

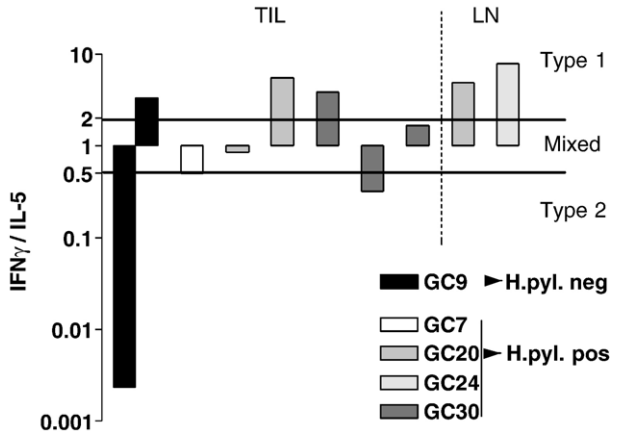

Figure 6 Polarization of tumor-specific T cell responses in TIL and LN-derived T cell cloids of gastric cancer patients. Tumor-specific release of type 1 (IFN-γ) and type 2 (IL-5) cytokines of T cell cloids from *H. pylori*-negative (n = 1) and *H. pylori*-positive (n = 4) patients were examined and IFN-γ:IL-5 ratios are depicted after subtraction of the non-specific cytokine release in the medium control. Tumor-specific responses with IFN-γ:IL-5 ratios higher than 2 were considered type 1 and ratios below 0.5 were regarded type 2. Tumor-specific responses with ratios between 0.5 and 2 were considered mixed type of responses.

CRAs have also been previously used as a read-out system to identify tumor-specific T cells in TVDLN and peripheral blood of patients with metastatic melanoma, renal cell cancer and metastatic breast cancer [30—32]. We were able to establish several gastric cancer cell lines and autologous mixed T cell cultures containing CD4+ and CD8+ T cells and tested these for tumor-specific cytokine release in CRAs. Tumor-specific T cells were identified in most of the tested T cell cloids as determined by the tumor-specific release of IFN-γ or IL-5. Moreover, most T cell cultures secreted both IFN-γ and IL-5 in a tumor-specific manner. It remains to be investigated whether IFN-γ and IL-5 are released by different T cell clones or by the same cells. The latter possibility has been described for human effector memory lymphocytes [33]. Effector memory T cells that are primed towards a type 1 polarization continue secretion of IFN-γ but can gain the ability to express IL-4 when stimulated under opposite polarizing conditions and vice versa. The T cell cloids studied here are derived from a relatively small number of T cells present in the initial 2 ml of TIL that were cultured in a single well of a 24-well plate and subsequently expanded. From experience, we generally expect that the IFN-γ and IL-5 detected from these cultures are secreted by a limited number of T cell clones. It would be interesting to determine the IFN-γ:IL-5 ratio at a single cell level by intracellular cytokine staining. However, we did not succeed in detecting intracellular IL-5 expression after tumor-specific stimulation.

The T cell cloids consisted of CD4 and CD8 cells. Therefore, cytokines could possibly be released from both cell types. However, we did not detect MHC class II expression on the tumor cell lines. Moreover, cytokine release after stimulation with fresh tumor samples, containing MHC class II expressing APC, could be blocked by the addition of an MHC class I blocking antibody. Therefore, we suggest that the cytokines are released by CD8-positive T cells. Additional studies analyzing tumor-specific intracellular IFN-γ secretion in CD4 and CD8 cells are in progress.

About 80% of the recruited patients were characterized to be *H. pylori*-positive. *H. pylori* causes continuous gastric inflammation in infected individuals. As a response to the attachment of the bacteria to the epithelial cells, neutrophils are recruited, followed by T and B cells, plasma cells, and macrophages. *H. pylori* infection is believed to induce gastric cancer and has been defined a class I carcinogen by the IARC [34]. In our group of patients, 11 patients (21.6%) had no signs of an active, ongoing or former infection with *H. pylori*. This percentage is comparable with the number of *H. pylori*-negative patients in other studies [35—38]. In a prospective study, we have analyzed the survival of gastric cancer patients after curative resection of the tumor. Interestingly, patients with a *H. pylori* infection had a significantly better 5-year overall survival (61.9%) than *H. pylori*-negative patients (19.2%) at median follow-up of 53 months (P = 0.002) [39]. Previously, Lee and co-workers showed similar results in a retrospective analysis [40,41].

What might explain these observations? Multiple studies have reported that *H. pylori* can polarize an immune response towards a type 1 cytokine profile [42—44]. In these studies, lymphocytes isolated from the stomach mucosa of *H. pylori*-positive patients produced IFN-γ but only low levels of IL-4 and IL-5. In addition, stomach mucosa biopsies of *H. pylori*-infected patients showed increased mRNA expression of the type 1 cytokines IFN-γ, IL-12, and IL-17 compared to stomach mucosa biopsies of *H. pylori*-negative patients. The mRNA expression of type 2 cytokines did not differ between these two groups [43]. Since tumor-specific type 1 cytokine responses are associated with protection from a tumor challenge and T-cell-mediated regression of established tumors [9], we hypothesized that the improved survival in *H. pylori*-positive patients may be secondary to the induction of a tumor-specific type 1 T cell response. Consistent with our hypothesis, cloid GC9 TIL3 from the only *H. pylori* negative patient studied, displayed a dominant type 2 cytokine response, secreting IL-5 and not IFN-γ upon stimulation with autologous tumor. A second cloid from the same patient secreted very little tumor-specific IFN-γ compared to the cloids derived from *H. pylori*-positive patients. The overall low levels of tumor-specific IFN-γ in T cell cloids from this one *H. pylori*-negative patient have limited value since no other cloids from *H. pylori*-negative patients were available. However, we consider that these preliminary findings are consistent with our hypothesis. Further, since tumor-specific type 2 responses have correlated with tumor progression [13], this could explain the decreased survival of *H. pylori*-negative patients after surgery compared to *H. pylori*-positive patients. Although most cloids derived from *H. pylori*-positive patients secreted high levels of IFN-γ, tumor-specific responses in all of these cloids were not exclusively type 1. It may be suggested that the level of tumor-specific cytokine secretion in cloids from *H. pylori*-positive patients reflects the differences in survival between these patients. While the number of patients analyzed in this study is not appropriately powered for such an analysis, it is tempting to consider that tumor progression

was secondary to the tumor successfully circumventing the type 1-polarizing capacity of *H. pylori*, resulting in the induction of a mixed or type 2 cytokine response. These observations have caused us to consider the possibility of infecting *H. pylori*-negative gastric patients with *H. pylori* prior to gastric resection. We postulate that the induction of a type 1 cytokine milieu in the stomach may promote the development of a tumor-specific type 1 response at the tumor site and may translate into improved survival rates for this subset of gastric cancer patients. This strategy, which may be considered similar to that employed for bladder cancer patients who receive intra vesicular BCG [45], would profit from the type 1 polarizing capacity of *H. pylori* with the advantage that the stomach would subsequently be resected, eliminating the target organ of the infection. This concept is further supported by preliminary preclinical studies suggesting that the addition of *H. pylori* to a tumor vaccine can substantially augment the tumor-specific immune response (C.H. Poehlein and I. Assmann, personal communication). Current efforts are expanding these studies to include a larger number of patients.

H. pylori-specific tumor-infiltrating lymphocytes have been described. It has been suggested that *H. pylori*-specific TIL provide help for B cell proliferation in patients with gastric B cell lymphoma of mucosa-associated lymphoid tissue[46]. In addition, *H. pylori*-specific T cells isolated from the lamina propria of *H. pylori*-infected individuals appeared to be predominantly CD4+ [47]. Thus, we hypothesize that TILs from *H. pylori*-positive gastric cancer patients also comprise *H. pylori*-specific T cells. A challenging approach would yet be to characterize whether these *H. pylori*-specific TILs are cross-reacting with tumor cells. Such a form of mimicry could be another explanation for the improved survival of gastric cancer patients with *H. pylori* infection after curative resection of the tumor.

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