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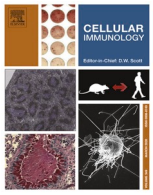

Higher prevalence of LAP+ (Latency TGFβ-Associated Peptide) T cells at the tissue level in patients with early gastric cancer

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ABSTRACT

The presence of cells with regulatory functions in patients with cancer is one of the mechanisms whereby the immune system cannot confront tumor growth. We sought to determine the prevalence of immunoregulatory *T*-cell subpopulations, expressing the latency TGFβ-associated peptide (LAP), in patients with gastric adenocarcinoma.

T cells were enriched from blood or gastric tissue (tumoral, TT or tumor-free, TF) samples from 22 patients, 6 with early (EGC) and 16 with advanced gastric cancer (AGC). CD4, CD8, LAP, FoxP3 and IFN-γ were measured by cytometry.

CD8 + LAP + cells were increased at tumoral sites, especially in early stages of the disease, as compared to tumor-free explants (EGC 5.28 % [4.67–6.64]*; AGC 2.90 % [1.37–4.44]; TF 3.14 % [2.33–4.16]; *p < 0.05 vs TF). Likewise, the LAP+/CD8 + LAP- ratio is increased in gastric samples from patients with early disease (EGC 0.38 [0.30–0.45]*, AGC 0.12 [0.07–0.14]; TF 0.12 [0.09–0.31]; *p < 0.05 vs AGC). Disease progression is accompanied by decreased LAP membrane expression and, probably, increased LAP secretion, therefore limiting the response to the tumor.

1. Introduction

Tumoral antigens may be targets of cells of the immune system. In fact, tumor-specific T cells are found in peripheral blood lymphocytes (PBL) or infiltrating the tumor tissue (tumor-infiltrating lymphocytes, TIL) in patients with cancer [1,2].

However, T lymphocytes from patients with advanced cancer may show poor immune responses [3]. Several mechanisms have been proposed to explain this malfunctioning, such as down-regulation of cell surface molecules of the CD3 complex, or development of an immunosuppressive milieu mediated by regulatory T cells (Tregs) [4]. Tregs will exert an effect on the interplay between tumoral and immune cells creating a tumoral microenvironment (TME) that will favor immunosuppression and tumor growth. Therefore, T lymphocytes and other immune cells do not exert this control and are then unable to keep tumoral cells at bay. Thus, assessing the status of these cells in cancerous lesions is relevant to the evolution of the disease.

The regulatory T cell subpopulation was initially identified by the expression of the CD4 + CD25high markers and represents 2 %-3% of all $CD4^+$ T cells. Later, the expression of the FoxP3 (*Forkhead box P3)* transcriptional factor was used to identify T cells with regulatory properties. FoxP3 associates to NF-AT and NF-κB nuclear factors and inhibits their ability to induce key cytokine synthesis. Thus, Treg cells can currently be identified as CD4 + $CD25^{high}$FoxP3 + cells, able to

Abbreviations: BMP1, Bone Morphogenetic Protein 1; FoxP3, Forkhead box P3; IFNγ, Interferon γ; LAP, Latency TGFβ-Associated Peptide; TGFβ, Transforming Growth Factor β.

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produce inhibitory cytokines, such as IL-10, TGFβ and IL-35 [5].

In addition, further FoxP3-, TGFβ-producing cell subpopulations with immunoregulatory functions have been reported [5,6]. These subpopulations express the latency TGFβ-associated peptide (LAP, a membrane-bound precursor of TGFβ) on their surface and secrete TGFβ. Thus, LAP identifies T cells with immunoregulatory properties [5]. Interestingly, detailed analysis of CD4 + CD39 + regulatory T cells in patients with head and neck squamous cell carcinoma (HNSCC) showed two subpopulations CD4 + CD39 + FoxP3 + CD25+, which mediates suppression of $CD4^+$ T_{conv} cells, and CD4 + CD39 + FoxP3- CD25-, which do not mediate such suppression. Upon 6 h. stimulation with SEB, only the suppressor subset became positive for markers characteristic of Treg cells, such as LAP [7].

TGFβ and LAP are the products of a single gene, which (after post-translational modifications and cleavage by furin convertase) remain non-covalently associated, forming the small latent complex (SLC). Ulterior processing of this complex results in activation of TGFβ via cleavage of LAP by non BMP1-like proteinases [8].

Increased numbers of Tregs, either CD4 + CD25+ [3] or FoxP3 + cells [9–11], have been described in patients or animal models with cancer, resulting in *T*-cell dysfunction and down-regulating the activity of effector functions against tumors.

Of note, it has been shown in colorectal cancer that infiltrating LAP^+ Treg cells are 50-fold more suppressive than conventional $FoxP3^+$ Tregs [12].

Our group has described several *T*-cell alterations in the function (low proliferative response, [13]) or phenotype (altered CD3ζ expression, [14,15]) in patients with gastric cancer, especially at the tumor site (gastric tissue) rather than in peripheral blood, although the underlying causative mechanisms remain unknown.

To further understand the cellular or molecular events underlying these *T*-cell alterations, we wished to study the prevalence of these immunoregulatory LAP + *T*-cells in a group of patients with gastric adenocarcinoma.

2. Patients, materials and methods

2.1. Patients

Peripheral blood and gastric tissue (tumor and tumor free) samples from 22 patients (14 men, 8 women, mean age: 67 yrs. Range 40 yrs.-85 yrs.) with gastric adenocarcinoma were obtained. Patients, submitted by the Department of General Surgery (Servicio de Cirugía General y Aparato Digestivo; Hospital Príncipe de Asturias, Alcalá de Henares), were classified according to the TNM criteria of the American Joint Commission on Cancer (AJCC) as follows: 6 stage I patients (early gastric cancer, EGC), 4 stage II, 9 stage III and 3 stage IV (the latter 16 considered as advanced gastric cancer, AGC). As a control, peripheral blood samples from 23 healthy (age and sex-matched) unrelated individuals were also included.

All the experiments were carried out with the approval of the Ethics Committee of both institutions involved in the study and all participants signed the pertinent informed consent.

2.2. Isolation of T cells

Blood samples and gastric biopsies of patients were obtained on the day of surgery.

Blood samples, whether from patients or control subjects, were collected in EDTA-containing tubes and processed using a *T*-cell enrichment cocktail (Stem cell technologies), following manufacturer's indications, achieving at least 98 % purity.

Tissue samples from patients, either tumoral (TT) or tumor-free (TF) tissue, were transported to the laboratory in culture media (RPMI 1640, Gibco) with 1 % antibiotic (100 U/mL penicillin and 100 μg/ml streptomycin). Samples were processed according to the protocol of Valle-Noguera et al., slightly modified [16]. Tissue was mechanically minced and incubated in Hank's Buffered Saline Solution (HBSS) 10 % FBS and 5 mM EDTA at 37 °C and shaken for 30 min. Next, it was incubated in digestion medium with collagenase IV (Gibco) at 37 °C and shaken for 1 h. Finally, the suspension was filtered through a 70 μm mesh and TILs and IELs isolated.

2.3. Flow cytometry

Surface (CD3, CD4, CD8, LAP) and intracellular (FoxP3, IFNγ) expression was assessed by cytometry using antibodies from Beckman Coulter (CD4-FITC, CD4-PE-Cy5, CD8-FITC, CD8-PE-Cy5 and CD3-APC), R&D Systems (LAP-PE) and e-Bioscience (FoxP3-PE-Cy5, FoxP3-APC and IFNγ-FITC). Labelling procedures were done following standard protocols. First, surface molecules were labelled by incubating cells (15 min, 4 °C, in the dark) with specific antibodies. After a washing step (600 g, 5 min), cells were prepared for intracellular staining, fixed (Cellfix solution, Becton Dickinson) for 10 min at 4 °C and permeabilized with 0.5 % BSA- 0.1 % saponin in PBS, and then incubated with the adequate antibodies (30 min, RT, in the dark). After two further washing steps, cells were resuspended in Facs Flow (Becton Dickinson), and finally subjected to cytometry.

To assess IFNγ expression, and prior to the staining step explained above, cells (2×10^6) were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) in the presence of brefeldin A (10 μg/ml) (all three from Sigma-Aldrich) for 4 h in culture media (RPMI 1640 with 10 % FCS, 1 % antibiotic and 1 % glutamine) in a CO_2 incubator.

Irrelevant, isotype-matched, monoclonal antibodies (Immunotech, Beckman Coulter) were used as negative controls. A minimum of 5000 CD4 + or CD8 + T cells was analyzed per sample and gated to exclude non-viable cells. Fluorescence intensities above the upper limit of the negative control distribution were considered positive.

2.4. Statistical analysis

Results obtained are shown as median value [interquartile range]. Mann–Whitney-two-sample test was used to compare groups. Significance was reached when a P-value less than 0.05 was obtained.

3. Results

3.1. Description of LAP + T cell populations in patients.

In the present cohort we found higher numbers of LAP-expressing cells in patients than in healthy subjects (not shown). Moreover, cytometry showed the presence in blood (Fig. 1) and tissue (supplementary Figs. 1 and 2) of CD4 + LAP + and CD8 + LAP + populations, clearly distinct from the FoxP3 + Treg cells.

In addition to expressing LAP, these cells produce IFNγ when properly stimulated with PMA and ionomycin for 4 h in the presence of brefeldin A (Fig. 2; supplementary Figs. 1 and 2). The already described IFNγ-dependent immunosuppressive function of CD8 + Treg cells [17], confidently assesses these LAP + populations as immunoregulatory ones.

CD4 + LAP + IFNγ-producing cells have been previously described in healthy human blood [6], while CD8 + LAP + cells have been reported in patients with systemic lupus erythematosus (SLE) [18].

3.2. LAP expression in patients depends on the disease stage

Patients were grouped according to the AJCC classification as presenting either early (stage I) or advanced (stages II, III and IV) cancer, and LAP expression analyzed in tissue-derived T cells. It is at the tissue level, rather than blood, where the role of LAP + cells seems more relevant.

Tissue-derived T lymphocytes were isolated upon surgery from

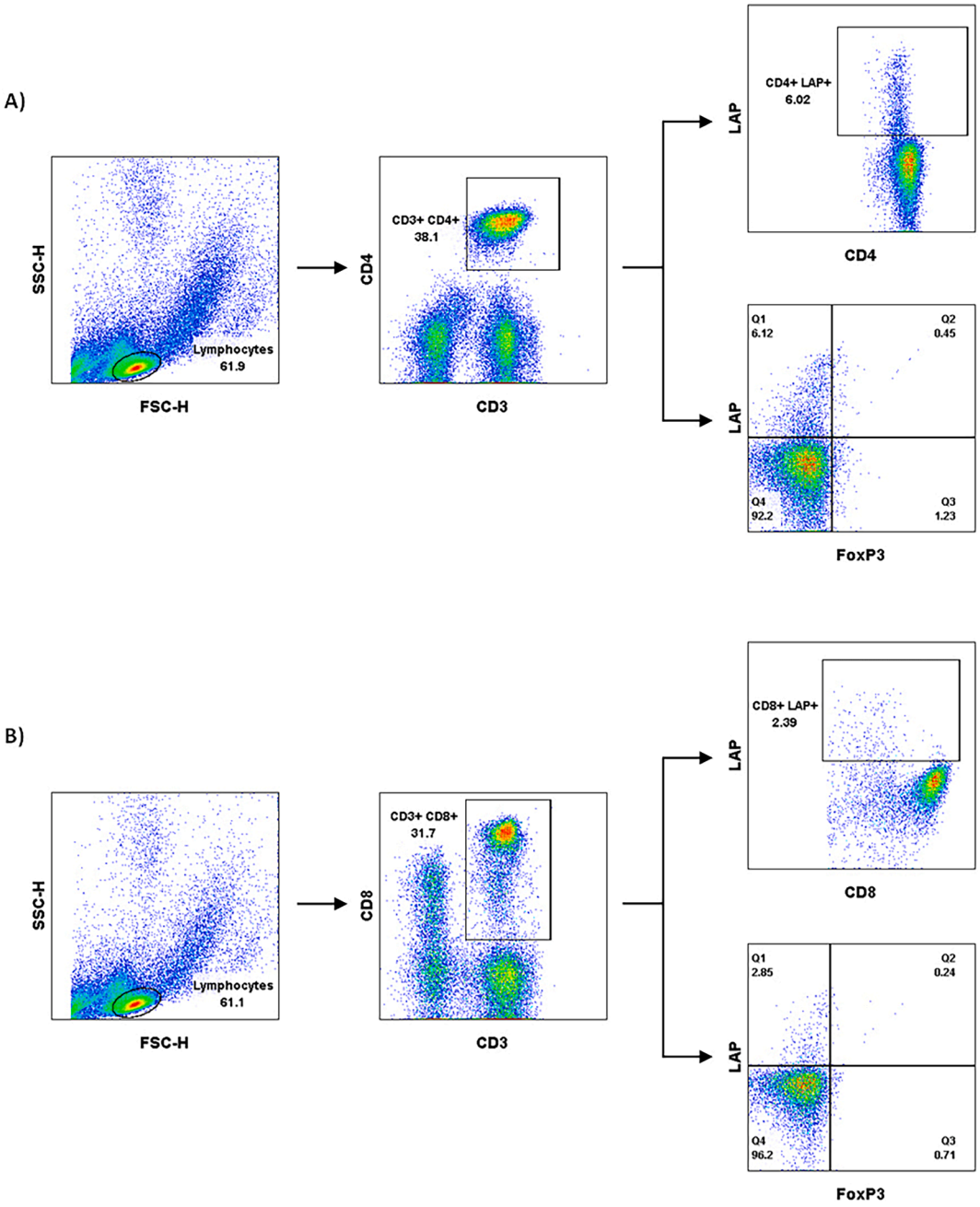

Fig. 1. Gating strategy of LAP+ and FoxP3+ CD4 and CD8 T cells in peripheral blood. (A) CD4 + LAP + and (B) CD8 + LAP + represent a subset of regulatory T cells distinct from FoxP3 + cells. Cytometry analysis disclosed two populations, CD3 + CD4 + LAP + and CD3 + CD8 + LAP+, phenotypically distinct from CD3 + CD4 + FoxP3 + and CD3 + CD8 + FoxP3 + regulatory T cells.

diverse types of sources: tumor free (TF, n = 11) or tumoral tissue (TT) sections from patients with early (n = 5) or advanced (n = 14) gastric cancer (Fig. 3). LAP expression was found in 13.3 % [8.8–15.1] of cells in early cancer (Fig. 3A) while much lower values were obtained in advanced disease or tumor-free samples (6.77 % [4.18–8.78] and 6.52 % [4.74–9.31], respectively), although significance was not reached. If we show LAP expression based on the helper or cytotoxic phenotype, the percentage of CD4 + LAP + cells (Fig. 3B) rose to 5.45 % [5.14–5.68] in early stage cancer samples compared to 3.90 % [1.87–5.13] and 3.38 % [2.12–4.78] in advanced cancer or tumor-free samples, respectively (N. S.). A similar pattern was found in CD8 + LAP + cells (Fig. 3C): 5.28 % [4.67–6.64], 2.90 % [1.37–4.44], 3.14 % [2.33–4.16] in early, advanced or tumor free samples, respectively, the latter significantly reduced when compared to early cancer ($p < 0.05$).

Since TF comes from the corresponding EGC or AGC samples, a paired T-test analysis of TF and EGC/AGC samples was carried out, yielding negative results in all instances ($p > 0.05$, not shown).

These data altogether show that LAP is more abundant in early cancerous lesions, and its expression decreases as the disease progresses, probably because LAP is converted to free, active TGFβ (see discussion).

3.3. *LAP+/CD8 + LAP- ratio*

Next, we wished to know whether differences could be found in the LAP+/CD8 + LAP- ratio. This ratio would reflect the immune suppression exerted by total LAP + T cells on cytotoxic CD8 + LAP- T cells. A high ratio suggests active suppression and thus a limited capability of the immune system (CD8 + cells) to confront tumor growth. Earlier

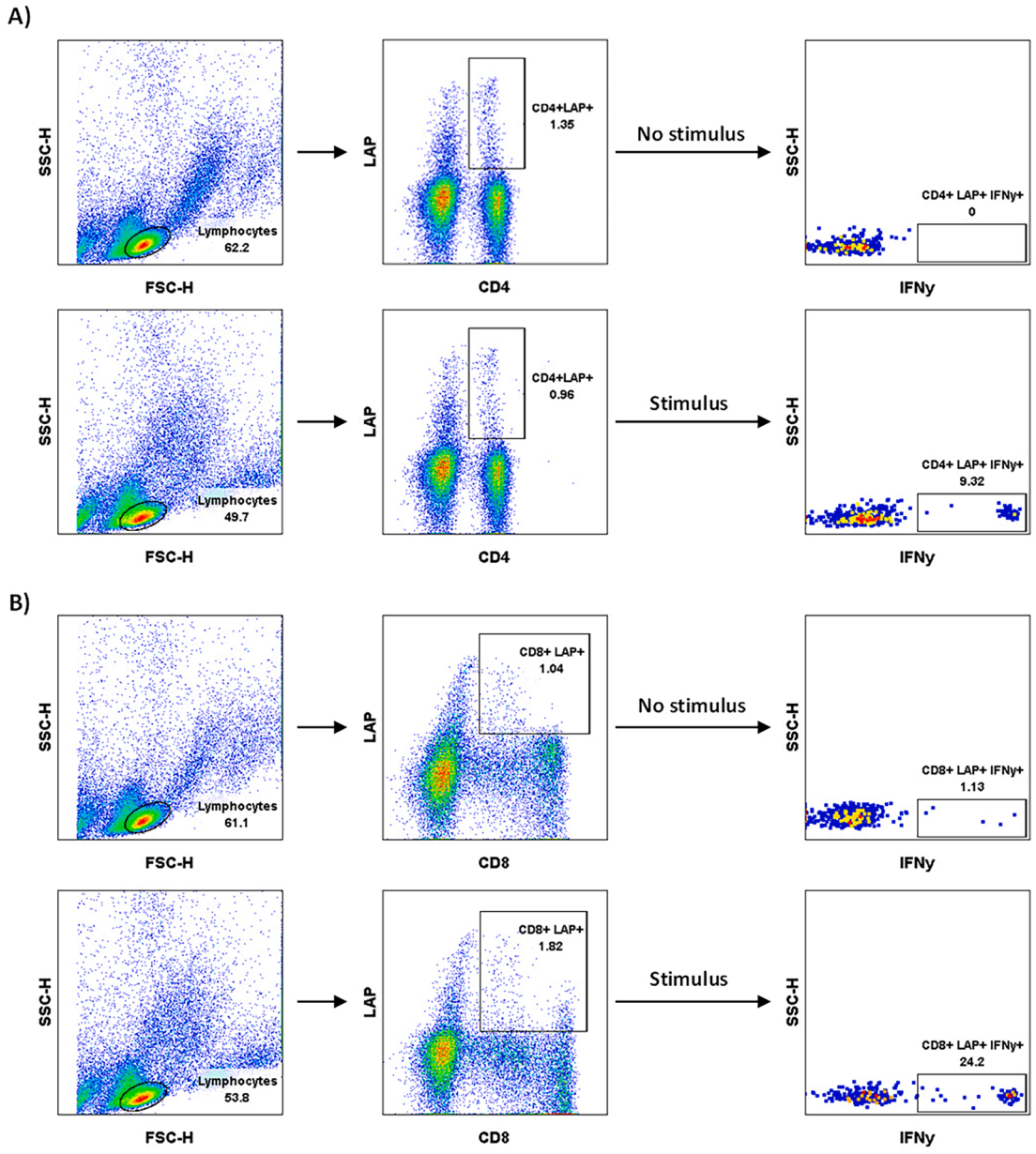

Fig. 2. CD4 + LAP+ (A) and CD8 + LAP+ (B) regulatory T cells secrete IFNγ upon stimulation. Blood T cells were isolated and stimulated with PMA, ionomycin and treated with brefeldin A, as indicated in the "Patients, materials and methods" section, and then labeled with antibodies against LAP, CD3, CD4, CD8, FoxP3 and IFNγ. Similar results were found with isolated tissue-derived populations (supplementary Figs. 1 and 2).

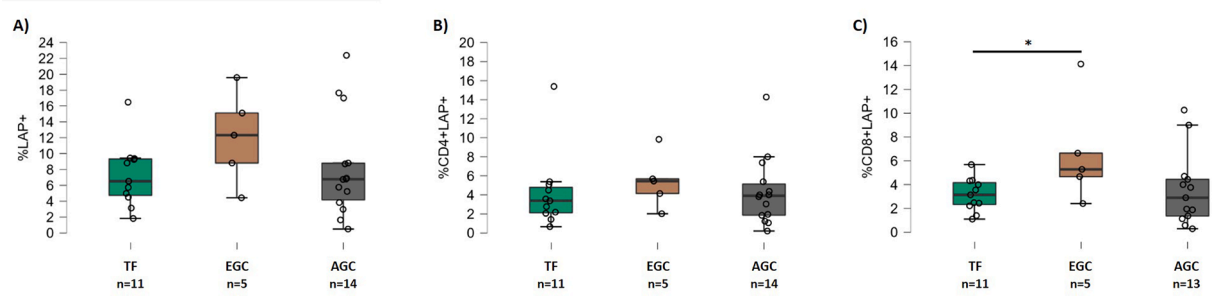

Fig. 3. Percentage of (A) total LAP + T cells, (B) CD4 + LAP + or (C) CD8 + LAP + cells in gastric tissue samples of patients with gastric adenocarcinoma, classified according to disease stage. T cells were isolated as indicated in the "Patients, materials and methods" section. TF: tumor free gastric samples; EGC: early gastric cancer; AGC: advanced gastric cancer. Boxplot. *$p < 0.05$. *n,* sample size.

published works have addressed this issue, using a ratio analogous to ours (FoxP3+/CD8 +) [11]. This was further described as a predictor of clinical benefit with PD1 blockade in non-small cell lung cancer patients, able to guide therapeutic decisions [19], and of poor disease prognosis [20].

Our analysis was done considering the disease status of the patients, and the results obtained are shown in Fig. 4. Significant differences are found between cells isolated from tumoral tissue samples from patients with early disease (0.38 [0.30–0.45]) or advanced cancer (0.12 [0.07–0.14]; $p < 0.05$). If, instead, cells from non-cancerous tumor-free lesions from patients were considered, no significant differences were then found (0.12 [0.09–0.31]). Measurement of this ratio at the tissue level makes most sense, since it is in this context where immune cells challenge tumor growth.

The increased ratio found can be explained both by higher numbers of LAP + cells in early cancer (see above) and by lower numbers of CD8 + cells, less abundant in tissue samples from early cancer (37.8 % [32.6–44.9]) than advanced disease (48.6 % [45.1–69.1]) or non-cancerous tumor-free tissue samples (47.2 % [29.2–60.4]) (not shown).

Hence, we suggest that patients in early disease stage show a suppressor phenotype (LAP +) that hampers CD8 + T cells ability to cope with the tumor. As the disease progresses, LAP is shed off the cell surface (see discussion), explaining the lower LAP expression in advanced disease.

4. Discussion

4.1. LAP + cells in human gastric adenocarcinoma

Cancer progression is challenged by CD8 T cells of the adaptive immune response. Regulatory T cells might be crucial in this regard, since they are able to down regulate the activity of immune cells [20] hampering their role in the control of tumors [21].

We measured the amount of Treg cells in blood or tissue-derived samples in individuals with gastric adenocarcinoma. LAP, rather than CD4 + CD25 + or FoxP3+, was used as marker of this population. LAP + cells can either be CD4+ (clearly distinct from classical CD4 + FoxP3 + Treg; Fig. 1; supplementary Figs. 1 and 2) [6] or CD8+, both with regulatory functions [17].

We found in our cohort higher numbers of LAP-expressing T cells in patients than in healthy subjects (not shown). This is consistent with previous works that also addressed the issue of Treg in cancer, assessed as FoxP3 + cells, reporting higher Treg numbers in patients [22].

The cells here described express LAP on their surface, can be either of the CD4 + or CD8 + phenotype, clearly different from FoxP3 + Treg cells, and secrete IFNγ when properly stimulated, pointing to a regulatory function for these cells (Fig. 2; supplementary Figs. 1 and 2). The increased frequency of LAP + CD8 + T cells with regulatory activity in tissue samples from early-stage gastric cancer patients, suggests their potential role in controlling the immune response to cancer, similar to what Mahalingam et al. described for CD4 + LAP + T cells in colorectal cancer (CRC) [23].

If patients were disclosed according to the disease status, LAP + cells were more abundant in early (stage I) than in advanced stages (stage II, III y IV) of the disease, and more than in tumor-free tissue samples (Fig. 3). This indicates that immunosuppression mediated by LAP + cells is more intense at the beginning of the disease and less so as the disease progresses. This finding is at odds with the results from other groups which report an increase in the number of immunoregulatory T cells (identified as CD4 + CD25 + cells) in advanced stages of tumor growth [3,22] or high proportion of infiltrating FoxP3 + Treg in the subset of patients with poor prognosis [10]. We think, however, there is no such contradiction, and the discrepancies reflect just the different measurement and kinetics of distinct cell populations with immunoregulatory properties. Moreover, it is known that TGFβ and IFNγ-producing CD8 + LAP + cells precede and up regulate the appearance of FoxP3 + cells [19], establishing a coordinate expression pattern.

LAP expression is induced by, among other factors, IL8 [6], a

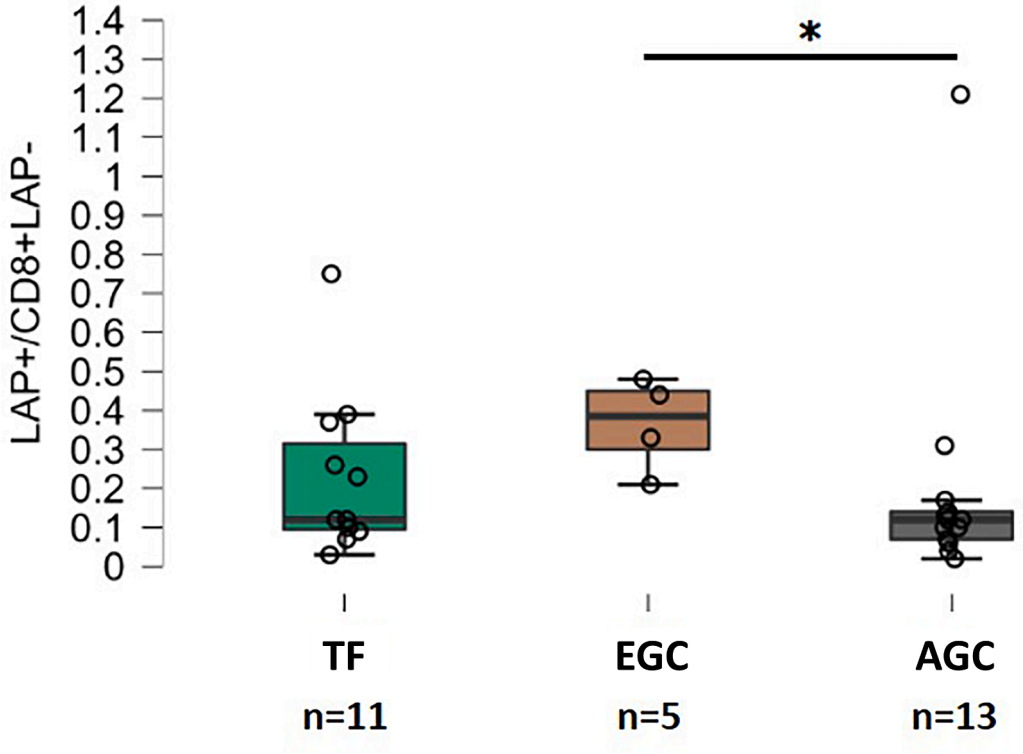

Fig. 4. LAP+/CD8 + LAP— ratio in tissue-derived T cells from patients with gastric adenocarcinoma, classified according to disease stage. T cells were isolated as indicated in the material and methods section. TF: tumor free gastric samples; EGC: early gastric cancer; AGC: advanced gastric cancer. Boxplot. *$p < 0.05$. *n*, sample size.

cytokine that can be readily produced by epithelial cells [24]. In initial stages of gastric cancer, the stomach epithelium may be sufficiently spared to produce elevated amounts of IL8, inducing LAP expression. As the disease progresses and the gastric epithelium is damaged, IL8 synthesis decreases and therefore LAP induction is also decreased; this, along with the secretion of LAP and conversion into active TGFβ, would explain the diminished LAP expression on T cells in advanced disease stages. In keeping, higher TGFβ levels are found in patients with advanced cancer [25,26] and blocking its function restores the immune system (cytotoxic T lymphocytes, CTL) ability to cope with the tumor [26].

4.2. LAP+/CD8 + LAP- ratio

This ratio is an indirect measurement of the immune regulation exerted by LAP + cells on CD8 cytotoxic cells. The higher the ratio, the more limited the ability of CTL to act on tumoral cells. In fact, its value is higher in the initial steps of the disease (Fig. 4). The ability of cytotoxic T cells to confront neoplastic cells is challenged by LAP + regulatory T cells. As the disease advances, the ratio diminishes, mainly because of a reduced number of LAP + cells. This diminished LAP expression is due to a conversion of membrane-bound LAP to active soluble TGFβ, able to keep the immune suppressive milieu required to allow tumor progression.

Other works have done a similar ratio measurement with different markers (FoxP3/CD8) [11], showing that intratumoral FoxP3/CD8 ratio proved to be an independent predictor of gastric survival: low or high ratio was associated to improved or diminished survival, respectively. These results may seem at odds with ours, but we think they are complementary. Assuming the sequential expression of LAP and FoxP3, a plausible hypothesis would be that in initial steps of the disease, when LAP is highly expressed and FoxP3 is almost absent, high LAP/CD8 and low FoxP3/CD8 ratios are expected. As the disease advances, LAP is freed from the plasma membrane and converted to TGFβ (increasing immune suppression), and FoxP3 is now induced, further exerting immune regulation functions at the tumor site.

Altogether, our results suggest that LAP+/CD8 + LAP- ratio, given its relevance at the initial moments of the disease, could be used as a marker to identify individuals requiring more aggressive therapy upon surgery. Furthermore, elimination of LAP + T cells is a conceivable approach to restore a functioning immune system, able to fight tumor back. In fact, a similar approach has been used in patients with autoimmune disease, such as systemic lupus erythematosus (SLE), where elimination of LAP + cells was associated with immunological exacerbation of the disease [18] and in animal cancer models, where the use of LAP-specific antibodies enhanced anti-tumor immunity [27]. Moreover, these data may set the rationale for developing new therapeutic strategies: published results reveal that regulation of TGFβ (with inhibitors, for instance) reverses the immunosuppressive status of the tumor microenvironment, a feature that might be useful for developing new immunotherapeutic strategies in cancer, such as a combination of immune-checkpoint inhibitors (ICI) and TGFβ inhibitors [26]. In this sense, it has been reported in lung cancer that targeting tumor α_v integrin to prevent TGFβ maturation, is an adequate approach for more effective ICI therapy [28].

5. Conclusion

In conclusion, the presence of T cells with immunoregulatory properties at the tumor site (LAP + cells in early stages of the disease and FoxP3 + cells later) may limit the ability of immune cells to react to neoplastic antigens, creating the adequate conditions for the cancer cells to expand unchallenged.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Authors Contributions

Ana Aguinaga-Barrilero, Ignacio Juárez, Christian Vaquero-Yuste, Marta Molina-Alejandre received tissue and blood samples from patients and carried out research experiments.

Alberto Gutiérrez-Calvo, Inmaculada Lasa, Adela López, Remedios Gómez, surgeons, operated on patients, obtained samples and followed them up.

José M. Martin-Villa. IP of the group designed the study and obtained funding.

All authors were involved in the writing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2022.104635.

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