ORIGINAL ARTICLE

Midgut carcinoid patients display increased numbers of regulatory T cells in peripheral blood with infiltration into tumor tissue

SOFIA VIKMAN1, ROBERTA SOMMAGGIO1, MANUEL DE LA TORRE2, KJELL ÖBERG3, MAGNUS ESSAND1, VALERIA GIANDOMENICO1,3, ANGELICA LOSKOG1 & THOMAS H. TÖTTERMAN1

1Division of Clinical Immunology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden, 2Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden and 3Department of Endocrine Oncology, Uppsala University Hospital, Uppsala, Sweden

Abstract

Introduction. Our aim was to investigate the immune status of midgut carcinoid patients. Cancer patients generally display suppressed Th1-type immunity that disables mounting of an efficient anti-tumor response. However, little is known about patients with neuroendocrine midgut carcinoids. Material and methods. Circulating regulatory T cells were determined in patient blood by staining for CD4, CD25 and FoxP3 in flow cytometric analysis. T cell proliferation was measured by Alamar Blue in response to polyclonal activation and the regulatory phenotype of patient CD25+/C27 cells was validated by allogeneic stimulation of CFSE labelled responders. Cytokine levels in patient peripheral blood were measured by ELISA and CBA. Tumor infiltrating T cells were analyzed by immunohistochemistry and immunofluorescence. Results. The results demonstrate that midgut carcinoid patients exhibit increased frequencies of circulating Tregs and patient T cells have a decreased proliferative capacity compared to healthy donors. Systemic Th1-promoting cytokines are reduced. Midgut carcinoid tumors display CD4+/C27 and CD8+/C27 T cell infiltration, always in the presence of regulatory CD4+/C27 FoxP3+/C27 cells. Discussion. Midgut carcinoid patients display elevated T regulatory cell numbers and T cell dysfunction. Therapeutic strategies to overcome tumor-induced Th1 immunosuppression are required in combination with anti-tumor vaccinations.

Cancer immunotherapy has thus far primarily been focusing on the activation and amplification of anti-tumor immune responses with emphasis on T helper (Th) 1 immunity activating cytolytic T cells (CTLs). A host of tumor-associated antigens (TAA) have been identified [1] and used in adoptive T cell therapies [2,3] and dendritic cell (DC) vaccinations [4-6]. Several clinical trials have been conducted, favourable clinical responses have been reported, however, complete responses have been rare [7]. Focus has now turned towards mechanisms of immune evasion, the tumor microenvironment and the Th1-immunosuppressive networks orchestrated by the tumor [8]. Presently, tumor-associated regulatory T cells (Tregs) receive much attention in this context. Tregs are crucial for maintaining peripheral tolerance against self-antigens but have also been described as a main obstacle hindering successful immunotherapy [9]. The most studied suppressor cell is the CD4+/C27 CD25+/C27 FoxP3+/C27 Treg cell that can be further subdivided according to cytokine profile etc [10,11]. Lately, it has become evident that effector T cells may transiently upregulate FoxP3. Since activated T cells express CD127, the CD4+/C27 Foxp3+/C27 CD127− population is considered to be the true Treg cell population [12,13]. Several studies report an increased prevalence of Treg cells in the peripheral blood and tumor tissue of cancer patients [14-19]. Increased Treg frequencies and tumor infiltration are correlated with reduced patient survival [20-24]. Interestingly, in a study on five patients with medullary thyroid carcinoma the number of Treg cells was decreased prior immunotherapy with DCs loaded with tumor-specific antigens [19]. In other trials, cytotoxic T lymphocyte antigen-4 (CTLA-4) blockade [25-27], low-dose cyclophosphamide [28] and the agent denileukin diftitox [29] have all been
used to target Tregs with a concomitantly enhanced clinical efficiency of anti-tumor immunotherapy.

Classical midgut carcinoids are well-differentiated neuroendocrine tumors arising from lower jejunum, ileum, caecum and ascending colon [30]. They are typically associated with overproduction of serotonin and large liver metastases may give rise to the carcinoid syndrome characterized by flushes, diarrhea and heart valve fibrosis [31]. The tumor has generally metastasized at the time of diagnosis, and surgery is seldom curative at that point. Medical therapy involving somatostatin analogues and interferon-α aim at relieving the symptoms of hormonal over-production and stabilize tumor progression [32]. Recently, immunotherapy has emerged as a novel potential treatment for neuroendocrine malignancies [6,33]. However, little is known about the immunological profile in these patients. Our work to develop an immunotherapeutic strategy for midgut carcinoid tumors [34,35] prompted us to investigate the immunological status including the presence of Treg cells in these patients.

**Material and methods**

**Patient material and healthy controls**

Patients (n=68) diagnosed with midgut carcinoid tumor at the Department of Endocrine Oncology, Uppsala University Hospital, Uppsala, Sweden were included in the study. Peripheral blood was obtained during routine disease monitoring and 39 of the included patients were treated with interferon α (IFNα) at the time of blood sampling. Permission to collect blood was approved by the regional ethical review board (ref.no 2005:241) and informed consent was obtained from each patient. All patients demonstrated metastatic disease and were divided into two groups based on their tumor burden. The distinguishing criteria for a high tumor burden was ≥5 liver metastases while patients with <5 liver metastases and/or local metastases in lymph nodes and mesentery and at extra-abdominal sites were considered to have a low tumor burden. Healthy blood donors (n=53) from Uppsala University Hospital Blood Center, Uppsala, Sweden were used as controls.

**Isolation and purification of blood cells**

Peripheral blood mononuclear cells (PBMCs) were obtained by means of ficoll density centrifugation (Ficoll-Paque PLUS, GE Healthcare, Uppsala, Sweden) according to the manufacturer’s instructions. Lymphocytes were enriched by plastic adherence for 2 hours. CD25⁺ lymphocytes were enriched by magnetic-activated cell sorting (MACS) using CD25⁺ Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

**Regulatory T cell quantification**

T cells expressing regulatory markers were quantified in patients and a group of healthy age-matched blood donors by surface staining of CD4-FITC and CD25-APC. Intracellular stainings of FoxP3 (APC/FITC) was performed with a kit from eBioscience (San Diego, CA, USA) with surface markers CD4-FITC or PerCP, CD25-APC, CD127-PE. Irrelevant isotype- and fluorescence-matched antibodies were used as controls and evaluated cells were gated on lymphocytes based on a forward and side scatter dot plot. All antibodies were from BD Biosciences (San Diego, CA, USA). Flow cytometry was performed on a FACS Calibur and FACS Canto (BD Biosciences).

**Proliferation assays**

T cell proliferation in response to stimulation with 1.5 μg/ml anti-CD3 antibody (OKT3 Orthoclone®, Janssen-Cilag, Cincinnati, OH, USA) and 50U/ml IL-2 (Proleukin®, Chiron, Emeryville, CA, USA) was measured using AlamarBlue™ (BioSource, Nivelles, Belgium) according to manufacturer’s instructions. Absorbance was measured at 24 hour intervals. Ten thousand stimulated PBMCs from patients carrying a high tumor burden and normal lymphocyte counts (0.7–3.9 × 10⁹/L) were compared to PBMCs of healthy blood donors during 6 days of culture in RPMI 1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin. All cell culture reagents were from Invitrogen (Carlsbad, CA, USA). Proliferation was measured similarly in the patient CD25⁺ and CD25⁻ cell fractions, respectively. Inhibition of proliferation by the CD25⁺ lymphocyte fraction from patients was assayed by labelling healthy donor lymphocytes with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) and stimulate them as described above. The CD25⁻ and CD25⁺ patient lymphocyte fractions were irradiated at 40 Gy and added to the stimulated healthy donor lymphocytes in different ratios on a 96-well plate. Cells were cultured for 5 days with fresh media and additional IL-2 on day 3. Cell proliferation was evaluated by FACS (FACS Calibur, BD Biosciences).

**Enzyme-linked immunosorbent Assay (ELISA)**

Serum levels of transforming growth factor beta TGFβ were measured by ELISA (Biosource, Nivelles,
Belgium) according to the manufacturer’s instructions. TGFβ was measured in serum from 25 patients recently diagnosed with midgut carcinoid and prior to any interferon treatment. Serum levels of TGFβ from 8 healthy blood donors were measured for comparison.

**Immunohistochemistry**

Tumor infiltrating lymphocytes were investigated by immunohistochemistry on formalin-fixed paraffin-embedded material from ten midgut carcinoid patients using mouse anti-human CD4 (Novocastra, Newcastle, UK) and mouse anti-human CD8 (DAKO, Glostrup, Denmark) antibodies. Sections were de-paraffinized and epitope retrieval was performed by microwave treatment in 10mM Tris-EDTA, pH 9.0. Endogenous peroxidase was blocked using Peroxidase blocking solution. Tissue sections were allowed to incubate for 1 hour at RT with primary antibodies followed by DAKO Envision Peroxidase and AEC Substrate Chromogen. All chemicals were purchased from DAKO. Stainings were evaluated by a pathologist using an Olympus BX40 microscope. The level of infiltrating immune cells within the tumor area was graded according to negative (−), scattered, single positive cells (+), scattered, single positive cells and reactive inflammatory foci (+ +), lymph node metastases (+ + +), which also served as a positive control. Lymphocytes not present in the immediate tumor area were disregarded.

**Cytometric bead array (CBA)**

Serum levels of interleukin-12p70, IL-1β, IL-10, IL-6, IL-8 were measured by CBA (BD Biosciences) according to the manufacturer’s instructions. Cytokines were measured in serum from 25 patients recently diagnosed with midgut carcinoid and prior to any interferon treatment and 18 healthy controls (10 healthy controls for IL1β).

Peripheral blood collected from midgut carcinoid patients and healthy blood donors was subjected to ficoll density centrifugation and stained for T cell markers. CD4⁺CD25⁺ lymphocytes, expressed as percentage of total CD4⁺, proved more abundant in patients compared to healthy controls (t-test, p = 0.0002). Patients had on average 3.3% CD4⁺CD25⁺ cells in comparison to 1.3% in healthy donors (Figure 1A, left panel). An example is shown in the right panel of Figure 1A. Intracellular staining of FoxP3 yielded even higher frequencies of Tregs, 6.5% in patients compared to 3.7% in healthy donors (t-test, p = 0.0054). The patients were subdivided into two groups carrying a high or a low tumor burden. There was a significantly higher proportion Tregs in patients with a high tumor load, 9.0%, compared to patients with a low tumor burden, 5.2% (t-test, p = 0.0166) (Figure 1B, left panel). An example is shown in the right panel of Figure 1B. Patients with a high tumor load were further analyzed regarding their CD4⁺CD25⁺CD127⁻ cell population. The CD4⁺CD25⁺CD127⁻ population was increased in patients compared to healthy controls, 4.9% versus 1.8% (t-test, p = 0.0320) (Figure 1C, left panel). An example is shown in the right panel of Figure 1C. The CD127⁻ population also contained a large fraction of FoxP3⁺ cells as opposed to the CD127⁺ population (Figure 1D).

**Results**

**Evaluation of the number of circulating Treg cells in patients with midgut carcinoid tumors**

The proliferative T cell response to polyclonal stimulation was tested in 10 patients with a high tumor burden. All patients displayed normal lymphocyte counts and were compared to age-matched healthy donors. Peripheral blood mononuclear cells (PBMC) were stimulated using an anti-CD3 antibody (OKT3) together with IL-2 and proliferation was measured using AlamarBlue. Proliferation was measured continuously for 6 days and the results showed that patient T cells do not proliferate as well.
Figure 1. Midgut carcinoid patients display increased Treg prevalence in peripheral blood. Patients display increased Treg prevalence based on (A) CD4⁺CD25⁺ stainings (patients n=46, healthy n=14) (B) CD4⁺FoxP3⁺ stainings (patients n=30, healthy n=21) (C-D) CD4⁺CD25⁺CD127⁻ stainings (patients n=12, healthy n=10) (p < 0.05). Evaluated cells were gated on the lymphocyte population based on a forward and side scatter dot plot and percentages have been calculated based on the total CD4⁺ population. Individual values are shown as filled circles and the mean value is indicated with a line. Representative FACS plots are shown, in the right panels of Figure 1A, B and C. (D) A significant fraction of the CD127⁻ population is FoxP3⁺ positive in comparison to the CD127⁺ population both in patients and healthy controls. FoxP3⁺ cells are labelled black in the dot plot shown in Figure 1C, compared to the grey Foxp3⁻ cells.

Evaluation of the systemic cytokine levels in cancer patients and healthy individuals

Serum levels of inflammatory and regulatory cytokines were analyzed in 25 patients with CBA and ELISA to make a general cytokine profile of these patients. Serum samples taken from patients were compared to serum from healthy controls (Figure 4). The results show a systemic environment with a significant reduction in the important CTL-promoting cytokines IL-12p70 and IL-1b (t-test, p < 0.05). The Treg-associated cytokine TGFβ was slightly increased, but not to a statistically significant degree. No differences were detected for IL-10 and IL-6. However, IL-8 was significantly increased in patients (t-test, p < 0.05).

Defining the presence of immune cells in biopsies from midgut carcinoid tumors

Infiltration of immune cells in the tumor area was determined by immunohistochemistry using anti-CD4 and anti-CD8 antibodies. Stained sections were evaluated and graded based on the presence of positive cells and reactive inflammatory foci inside the tumor area. The results show that these tumors are infiltrated both by CD4⁺ and CD8⁺ T lymphocytes with a tendency towards heavier infiltration in metastases compared to primary tumors. The complete evaluation of CD4⁺ and CD8⁺ T cell infiltration is

as healthy donors (Figure 2A). Separating patient PBMCs into a CD25⁺ and CD25⁻ fraction and measuring proliferation showed that the CD25⁺ fraction is highly resistant to polyclonal activation. The CD25⁻ fraction remained suppressed compared to healthy donors even when the CD25⁺ fraction had been removed (Figure 2B).

Functional analysis of patient Treg cells

To validate whether the patient CD25⁺ fraction contains cells that are truly regulatory in nature, a proliferation test was performed using CFSE-labelled lymphocytes from a healthy donor. Labelled responder cells were stimulated with anti-CD3 antibody (OKT3) and IL-2 and patient CD25⁺ and CD25⁻ lymphocytes were added in different ratios. The cells were cultured for 5 days before FACS evaluation of CFSE dilution. Labelled responder cells mixed with patient CD25⁻ cells in a 1:1 ratio proliferated vigorously as opposed to cells mixed with patient CD25⁺ cells (Figure 3). Labelled responder cells mixed with patient CD25⁺ cells displayed inhibited cell division and a CFSE fluorescence profile similar to unstimulated responder cells both at a 1:1 and 10:1 ratio. This experiment was repeated using blood from three patients and three healthy controls with the similar results. These results validate that patient CD25⁺ lymphocytes do contain cells with regulatory properties.
midgut carcinoid patients. In our study, the levels of tryptophan in the patients were not investigated but preliminary data from reverse transcription PCR and microarray studies indicate that IDO mRNA is expressed in the midgut carcinoid tumor area (data not shown) and this might contribute to the increased systemic levels of Tregs seen in these patients. Midgut carcinoid patients displayed increased circulating levels of Tregs based on CD4\(^+\)CD25\(^+\) and CD4\(^+\)FoxP3\(^+\) staining. The differences proved statistically significant in relation to healthy donors. Treg frequency also increased with the tumor load. A group of patients carrying a high tumor burden, defined by 5 liver metastases or more had a significantly higher proportion FoxP3\(^+\) cells in peripheral blood compared to patients carrying a low tumor burden. There was no clear cut correlation between interferon treatment and high presence of Tregs, however patients with extremely high Tregs levels (>10% CD4\(^+\) FoxP3\(^+\)) cells were all receiving IFN\(\gamma\) treatment. Additional experiments need to be performed to further discriminate between high tumor burden and interferon treatment. As previously shown by other investigators [12,13] the FoxP3\(^+\) cells were mainly located in the CD4\(^+\)CD25\(^+\)CD127\(^-\) cell fraction and there were no difference between patients and healthy donors in this regard. The cell population CD4\(^+\)CD25\(^+\)CD127\(^-\) containing the highest proportion FoxP3\(^+\) cells was also increased in patients. A regulatory cell population primarily defined by cell surface markers could be of importance for sorting purposes.

Patient T cells were less responsive to polyclonal activation with OKT3 and IL-2 compared to healthy donors. Proliferation measured by AlamarBlue after stimulation was significantly lower among patients compared to healthy individuals. An explanation for

**Discussion**

Numerous reports have described cancer patients as immunosuppressed with high numbers of circulating and tumor infiltrating Tregs. Our data confirm this to be the case also for midgut carcinoid patients. Midgut carcinoid primary tumors develop in the gut, a T\(_{H1}\)-suppressed environment, rich in Tregs and TGF\(\beta\) [36–38]. The tumors metastasize most commonly to mesentery and liver. Previous investigations indicate that midgut carcinoid tumors can produce TGF\(\beta\) [39–43] and the excessive serotonin synthesis by tumor cells can give rise to decreased systemic levels of tryptophan [44]. Tryptophan is an essential amino acid that is the substrate for all serotonin synthesis within the CNS and the enteric system [45]. Tryptophan can also be degraded by indoleamine 2,3 dioxygenase (IDO) along the kynurenin pathway and IDO-expressing APCs have been implicated in T cell suppression. Depletion of tryptophan has been shown to induce T cell anergy, and in combination with tryptophan catabolites, it may induce T cells with a regulatory phenotype [46,47]. Decreased systemic levels of tryptophan could contribute to immunosuppression in midgut carcinoid patients. In our study, the levels of tryptophan in the patients were not investigated but preliminary data from reverse transcription PCR and microarray studies indicate that IDO mRNA is expressed in the midgut carcinoid tumor area (data not shown) and this might contribute to the increased systemic levels of Tregs seen in these patients.

**Figure 2.** Patient PBMCs are less responsive to polyclonal T cell activation compared to healthy controls. (A) Patient PBMCs (n = 10) proliferate poorly in response to polyclonal activation with OKT3 and IL-2 in comparison to healthy controls (n = 10, p < 0.02). Proliferation of triplicate samples was measured continuously for 6 days with AlamarBlue. Mean absorbance values at indicated days are plotted with SD. (B) The CD25\(^+\) fraction of patient lymphocytes are highly resistant to polyclonal stimuli. The CD25\(^-\) fraction remains hyporesponsive after removal of the CD25\(^+\) population and do not reach mean values for healthy donors, shown in Figure 2A. Mean absorbance values at indicated days for the CD25\(^+\) and CD25\(^-\) populations from 5 patients are plotted with SD.

Presented in Table I. Another set of frozen tumor specimens were used to detect presence of FoxP3\(^+\) expressing CD4\(^+\) cells in the tumor area with immunofluorescence. An initial staining with solely CD4\(^+\) revealed presence of CD4\(^+\) cells in 2 of 4 primary tumors and 4 of 6 metastases. These tumors were further stained with CD4\(^+\) and FoxP3\(^+\) and double-positive cells were detected in all cases. The complete evaluation of CD4\(^+\)FoxP3\(^+\) T cell infiltration is presented in Table II. Representative immunohistochemistry and immunofluorescence stainings are shown in Figure 5.
this could be the high Treg content in patient blood. Separating patient PBMCs into CD25+ and CD25- cells revealed an extremely low proliferative capacity within the CD25+ cell fraction, while the CD25- cell fraction displayed higher proliferation. The CD25+ cell fraction did, however, not reach the proliferative capacity seen in healthy individuals, suggesting that they remain in an anergized state.
The most important characteristic of Tregs are their ability to suppress proliferation in other cells. By separating patient PBLs into CD25$^{+}$/CD27$^{-}$ and CD25$^{+}$/CD28$^{-}$ cells and adding these populations to CFSE-labelled, OKT3-activated healthy donor lymphocytes the CD25$^{+}$/CD27$^{-}$ cell fraction could effectively inhibit allogeneic proliferation while the CD25$^{+}$/CD28$^{-}$ cell fraction could not. This experiment proves that the CD25$^{+}$/CD27$^{-}$ cell fraction contains cells with regulatory properties and an increased CD25$^{+}$/CD27$^{-}$ population might lead to decreased T cell expansions in patients. Further, patients showed a tendency towards Th1-suppression based on the general cytokine profile in serum. The proinflammatory cytokines IL-12p70 and IL-1$\beta$ were significantly reduced in patients and TGF$\beta$ was slightly increased. However, differences in cytokines may in general be difficult to detect systemically and might be more obvious in the tumor microenvironment.

An investigation of the tumor area revealed T cell infiltration in a majority of cases using CD4 and CD8 antibodies. T cells infiltrating the immediate tumor area were analyzed whereas T cells located in the outer surroundings were not considered. In general, metastases were more heavily infiltrated by T cells than primary tumors suggesting an activation of the immune system during tumor progression. Scattered cells and reactive foci appeared with both CD4 and CD8 stainings. Immunofluorescence with double staining of CD4 and FoxP3 revealed presence of Treg-like cells in all cases infiltrated by CD4$^{+}$ cells. These tumor-infiltrating Tregs may contribute to suppression of infiltrating anti-tumor effector T cells.

Our analysis shows that patients with midgut carcinoid tumors are immunosuppressed with increased systemic prevalence of Tregs and Th1-suppressive cytokines. Patient T cells have decreased...
proliferative capacity and the tumor area shows infiltration of T cells in combination with Treg-like cells. This has to be considered when creating an immunotherapeutic strategy for this type of malignancy. In coming studies, different strategies to block immunosuppression by Treg cells will be investigated.

Table I. Tumor infiltrating CD4$^+$ and CD8$^+$ cells.

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<th>Midgut Carcinoid Origin</th>
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− negative.
+ single positive cells scattered within the tumor area.
++ reactive foci and single positive cells within the tumor area.
+++ multiple positive cells, i.e. lymph node metastases.

Table II. Tumor infiltrating CD4$^+$FoxP3$^+$ cells.

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Figure 5. Midgut carcinoids demonstrate lymphocyte infiltration including FoxP3$^+$ cells. Presence of tumor infiltrating lymphocytes was detected by immunohistochemistry (n = 10) and immunofluorescence microscopy (n = 10). Inflammatory foci with CD4$^+$ and CD8$^+$ cells in the tumor area are shown at 20x magnification. A representative immunofluorescent staining of FoxP3$^+$-expressing CD4$^+$ cells, with CD4$^+$ indicated by red and FoxP3$^+$ by green, is also shown.
Acknowledgements

The authors would like to thank research nurses Monica Hurtig and Lena Olsson at the Clinic for Endocrine Oncology, Uppsala University Hospital for kindly providing patient samples. The authors would also like to thank Lisa H. Ekborn for laboratory assistance and Jan Grawé for excellent technical assistance with immunofluorescence microscopy. Finally, the authors would like to express their gratitude towards the Verto Institute and Dr. Raymond and Beverly Sackler for scientific and financial support.

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