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Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors

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We have made a detailed inventory of the immune infiltrate of gastrointestinal stromal tumors (GISTs), which originate from mesenchymal cells in the intestinal tract. These sarcomas are heavily infiltrated with macrophages and T cells, while immune cells of other lineages were much less abundant. Dissecting the functional subtypes of T cells with multicolor fluorescent microscopy revealed substantial populations of cytotoxic T cells, helper T cells and FoxP3$^+$ regulatory T cells. The balance of cytotoxic T cells and FoxP3$^+$ T cells was toward immune suppression. Analysis of the macrophage population also showed a dominance of anti-inflammatory cells, as the M2 type scavenger receptor CD163 was abundantly present. Other subsets of macrophages (CD14$^+$CD163$^-$) were occasionally detected. M2 type CD163$^+$ macrophages were associated with the number of infiltrating FoxP3$^+$ regulatory T cells and twice as many macrophages were found in metastatic GIST compared to primary lesions. Most metastatic GISTs had been treated with the tyrosine kinase inhibitors imatinib and sunitinib, but the high macrophage infiltrate was not related to this treatment. However, imatinib and sunitinib did induce secretion of anti-inflammatory IL-10 in macrophage cultures, indicating that treatment with these inhibitors might contribute to an immune suppressive microenvironment in GIST. Overall, our data reveal a picture of GIST as an active site of tumor-immune interaction in which suppressive mechanisms overrule potential antitumor responses. Tyrosine kinase inhibitors might promote this negative balance.

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract, with an annual incidence of around 20 per million.[1] These sarcomas are most likely derived from the interstitial cells of Cajal, which are the pacemaker cells of the intestine or their precursors.[2] The most common sites of origin are the stomach (60%) and small intestine (25%); whereas primary tumors in colon, rectum or esophagus are rare.[3] GISTs primarily metastasize to liver and peritoneum, although metastatic lesions in lung, bone, cerebrum, lymph nodes and skin have been described.[4] The vast majority of GISTs expresses the surface receptor cKIT (stem cell factor receptor, CD117).[1] Activating mutations in this tyrosine kinase receptor are in fact a hallmark of this sarcoma and thought to represent an early oncogenic event.[5] GISTs with wild-type cKIT sequence harbor often activating mutations in another tyrosine kinase receptor, the platelet-derived growth factor receptor A (PDGFRA).[6] The activating mutations in the signal transducing domains of these receptors result in ligand-independent powerful transforming signals, convincingly illustrated in transgenic mouse models, in which several different mutations in cKIT recapitulated GIST disease.[7,8] Recruitment of additional oncogenic pathways is only observed in later stages of the disease.[9]

The critical involvement of the tyrosine kinase receptors in the development of this sarcoma was instrumental to an effective treatment. Structural design of blocking ATP analogs that would selectively bind in the pockets of these kinases made several small-molecule drugs available for treatment of GIST.[10] FDA-approved therapeutics that are used for first line treatment of GIST are imatinib mesylate (Gleevec/Glivec) and in case of refractory disease, sunitinib malate (Sutent). Although quite specific, these inhibitors also target other tyrosine kinases. Of the two mentioned, sunitinib has the

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broadest profile being capable to inhibit cKIT, PDGF, VEGF receptors, Flt3 and the M-CSF receptor.[11–14] cKIT and the M-CSF receptor are important for the hematopoiesis and differentiation of several immune cells, especially in the myeloid lineage. The effect of these FDA-approved drugs on immune cells *in vivo*, however, is less well studied. Imatinib indeed dampens the development of monocytes and macrophages from $CD34^+$ bone marrow stem cells *via* blocking of the M-CSF receptor.[15–18] In general, patients do not suffer from severe deprivation of the myeloids at the doses used; although recently 1 study reported a significant decrease of absolute monocyte counts in sunitinib treated patients.[19] The effect on lymphocytes appears to be much less pronounced. T cell proliferation can be inhibited *in vitro* by imatinib,[20,21] but the generation of antigen-specific T cell responses *in vivo* is not hampered.[22,23]

To examine the impact of imatinib and sunitinib treatment on immune cells in GIST, we started with an in-depth analysis of the tumor immune infiltrate in primary and metastatic lesions. We found very high numbers of T cells and macrophages. To determine the subtype of these lineages, we performed 3-color fluorescent staining to distinguish cytotoxic T cells, helper T cells and regulatory T cells. The regulatory T cells were determined by nuclear FoxP3 staining. $FoxP3^+$ T cells are immunosuppressive and have been associated with a broad range of tumors.[24,25] The presence of intratumoral regulatory T cells and especially a negative balance with cytotoxic T cells is indicative for a poor survival in most cancers,[25] suggesting that $FoxP3^+$ T cells actively suppress immune responses against the tumor. Macrophages can be divided on the basis of their functions: classically activated macrophages (often referred to as M1) are inflammatory and can mediate cytotoxic responses against tumors and intracellular pathogens. Alternatively activated macrophages (typically named M2) are anti-inflammatory and are important for tissue repair responses.[26,27] In this study, we analyzed the immune cell subtypes of T cells and macrophages that infiltrated GISTs and the relationship with the therapeutically applied tyrosine kinase inhibitors.

Table 1. Characteristics of the gastrointestinal stromal tumors in this study

	Primary	Metastatic
Total number of cases	28	19
Location		
Stomach	16	3
Small and large intestine	10	8
Liver	0	3
Other	2	5
Sex		
Male	18	10
Female	10	9
Treatment with kinase inhibitors[1]		
No	24	6
Yes	4	13

[1]Patients received imatinib alone or imatinib with subsequent sunitinib.

Material and Methods

Patient characteristics and material

Paraffin-embedded GIST samples of 47 patients at the Leiden University Medical Centre from 2004 to 2009 were included in this study. Diagnosis of GIST was confirmed for all samples. Characteristics of the patients are shown in Table 1. The cell type of GISTs was determined as 23% epitheloid cells, 68% of spindle shaped cells and 9% of a mixture of both cell types. The use of clinical material was approved by the institutional review board according to the guidelines of the Dutch Federation of Medical Research Associations.

Enzymatic and fluorescent immunostaining of infiltrating immune cells and Human leukocyte antigen class I

All stainings on tissue sections were performed on a 4-μm formalin-fixed and paraffin-embedded tumor material. The human leukocyte antigen (HLA) class I staining and quantification using the mouse monoclonal antibodies HCA2 and HC10 (anti-HLA-A and anti-HLAB/C, respectively; Dr. J. Neefjes, Netherlands Cancer Institute, Amsterdam, the Netherlands) and the primary rabbit polyclonal anti-β2m (A 072; Dako, Glostrup, Denmark) was performed on citrate antigen retrieval-treated sections, as previously described.[28] The results were scored according to the Ruiter system, in which percentages and intensities of cells are taken into account to determine groups with different expression levels.[28] The used antibodies for immune cell detection on ethylenediaminetetraacetic acid (EDTA)-antigen retrieval sections were anti-CD68 (KP1; Dako), anti-CD163 (10D6; Novocastra, Valkenswaard, Netherlands), anti-CD1a (ICA04; Neomarkers, Fremont, CA), anti-CD20 (L26; Dako), S100 (cow/s100; Dako). Citrate-treated sections were stained with anti-NKp46 (Mab1850; R&D, Abingdon, United Kingdom) and anti-HLA-DR (TAL.1B5; Dako), and trypsine-treated sections were stained with anti-CD208 (DC-LAMP, 104G4; Immunotech, Praha, Czech Republic). All sections were then incubated with PowerVision-Poly/HRP directed against rabbit, rat and mouse IgG (Dako) and a 3,3'-diamino-benzidine-tetrahydrochloride (DAB) solution (0.125 g). Two researchers independently counted the number of antigen-stained cells on 10 representative high power fields per slide. For studying the amount of $CD163^+$ cells, 10 photographs were made per slide and after transformation to black and white images, the total amount of black pixels, representing $CD163^+$ cells was measured with ImageJ (W. Rasband, National Institutes of Health) and expressed as percentage pixels of the total photograph.

Simultaneous detection of CD3, CD8 and FoxP3 was performed with 3-color fluorescence staining on deparaffinized and citrate-treated sections using a mixture of anti-CD3 (rabbit polyclonal, ab282, Abcam, Cambridge, MA), anti-CD8

Table 2. Immune cell infiltrate in gastrointestinal stromal tumors categorized to lineage markers

	Lymphocytes			Myeloid cells			
	T cells	B cells	NK cells	Macrophages	Dendritic cells		
Lineage surface marker	CD3	CD20	NKp46	CD68	S100	CD1a	CD208
Number of cases	44[1]	11	11	30	30	11	11
Median	117.6[2]	0.8	0.08	211.7	15.6	0.3	0.1
25th percentile	51.7	0.5	0	100.1	9.2	0.2	0
75th percentile	343.9	5.2	0.1	271.6	21.9	3.4	0.3
Standard deviation	253.1	6.3	2.8	142.8	13.9	2.5	0.8

[1]Number of GIST samples included for this marker. [2]Values are expressed as number of cells per mm^2.

(mouse monoclonal IgG2b, 4B11, Novocastra) and anti-FoxP3 (mouse monoclonal IgG1, 236A/E7, Abcam). The epitopes were visualized with fluorescently labeled second step antibodies goat-anti-rabbit IgG-Alexa Fluor 546, goat-anti-mouse IgG2b-Alexa Fluor 647 and goat-anti-mouse IgG1-Alexa Fluor 488 (Molecular Probes, Breda, Netherlands). Images were captured with a confocal laser scanning microscope (LSM510, Zeiss, Sliedrecht, Netherlands) in a multitrack setting and were analyzed as previously described.[28] Fluorescent staining for CD14, CD33, CD163 and HLA-DR were similarly performed using combinations of anti-CD14 (mouse IgG2a, clone 7, Abcam), anti-CD33 (mouse IgG2b, clone PWS44, Novocastra), anti-CD163 (mouse IgG1, 10D6, Novocastra) and anti-HLA-DR (mouse IgG2a, clone TAL.1B5, Abcam) antibodies with different combinations of secondary fluorescently labeled antibodies goat-anti-mouse IgG2a-Alexa Fluor 546, goat-anti-mouse IgG2b-Alexa 647 and goat-anti-mouse IgG1-Alexa Fluor 488 (Molecular Probes).

In vitro macrophage cultures and assays

All macrophages were generated as previously described by us.[29] Briefly, monocytes from anonymous healthy donors' buffy coats were enriched for CD14 expression with MACS microbeads (Miltenyi Biotech) and cultured in the presence of 5 ng/mL GM-CSF (Invitrogen-BioSource) or 50 ng/mL M-CSF (R&D Systems) for 6 days to generate M1 and M2, respectively. Cytokine secretion was induced by 100 ng/mL lipopolysaccharide (LPS) for 16 hr and measured with enzyme-linked immunosorbant assay (ELISA) (Invitrogen-BioSource). Imatinib mesylate and sunitinib malate were purchased from Axon Medchem BV (Groningen, The Netherlands) and added to the monocyte cultures at the start in different concentrations, as indicated in the figures. Flow cytometry analysis was performed to validate the macrophage purity and differentiation using CD1a, CD14, CD163, CD80, CD86 and HLA-DR. All cultures were more than 95% pure and no toxicity by the tyrosine kinsase inhibitors was detected at the applied concentrations. We observed loss of cells in the cultures with concentrations higher than 1.25 μM for imatinib and 0.019 μM for sunitinib. Cocultures with allogeneic peripheral blood mononuclear cells (PBMC) was described previously.[30] In short, allogeneic PBMC were cultured with M1 or M2 type macrophages in the presence of 300 nM imatinib for 2 weeks with exogenous IL-2 (25 U/mL) and IL-15 (10 ng/mL). T cell lines were rested for 2 days without exogenous cytokines before analysis with flow cytometry.

Statistical analysis

Values for the count of immune cells were expressed as number of cells per mm^2. None of the data on infiltrate counts followed a normal distribution. Correlations between different types of immune cells were tested with the nonparametric Spearman's rank analysis. The nonparametric Kruskal-Wallis test was used to investigate different number of immune cells between clinical parameters, and to compare 2 categories the Mann-Whitney test was used. All statistical analyses were performed with the statistical package for social sciences software package 16 (SPSS). Two-sided p values of <0.05 were judged to be significant.

Results

GISTs are heavily infiltrated with immune cells

Characteristics of the included 47 tumor samples in this study of confirmed GIST are displayed in Table 1. Forty percent of the samples was derived from metastatic lesions and most of these had been treated with imatinib and sunitinib previous to resection. The vast majority of the GISTs was located in stomach and small intestine, some metastases were removed from the omentum ($n = 4$) and liver ($n = 3$). Spindle-cell–type and epithelial-cell–type tumors were equally represented in the collection. To determine the type of immune cells in the infiltrate of GIST, we stained the samples with several lineage-specific markers using immunohistochemistry and counted the number of cells per power field and calculated these numbers to cells per mm^2. Strikingly high numbers of macrophages and T cells were detected in most GIST samples, as determined with antibodies against CD68 (median of 211.7 $\pm$ 142.8 cells/mm^2) and CD3 (median of 117.6 $\pm$ 253.1 cells/mm^2), respectively (Table 2). In contrast, hematopoietic cells from other lineages, B cells, natural killer (NK) cells and dendritic cells were generally much less frequent or even absent in GIST (Table 2). We excluded immune cells that were clustered adjacent to vasculature and in necrotic areas, to focus on immune cells that have direct

Table 3. Loss of HLA class I molecules in GIST samples[1]

	Negative	Intermediate	Normal
β2m	0/30 (0%)	10/30 (33%)	20/30 (67%)
HLA-A	12/31 (39%)	9/31 (29%)	10/31 (32%)
HLA-B,-C	6/30 (20%)	8/30 (27%)	16/30 (53%)
HLA overall[2]	6/30 (20%)	15/30 (50%)	9/30 (30%)

[1]Samples were scored for intensity and surface area of staining, as described in Material and Methods. [2]For the values HLA overall, scores of both antibodies were combined.

and close contacts to tumor cells. Thus, the immune infiltrate of GIST is characterized by macrophages and T cells.

Expression of HLA molecules is frequently lost in GIST

HLA class I molecules are tissue antigens expressed by all nucleated cells in the body and function as ligands for T cells. Solid tumors regularly downmodulate these molecules and thereby avoid recognition by tumor-directed T cells.[31,32] Most analyses on HLA class I loss concerned carcinomas and the status of these molecules in sarcomas, including GIST is much less studied. We stained GIST samples with two different antibodies, one specific for HLA class I molecules from the HLA-A locus and one specific for proteins from the HLA-B and -C loci, to be able to detect partial and allelic loss. Complete lack of HLA-A was detected in 38% of the GISTs, whereas HLA-B and -C molecules were absent in 20% of the samples (Table 3). Infiltrating immune cells still stained positive in these cases, but the tumor cells were otherwise completely negative. Absence of the different HLA proteins was concomitant, in that the 20% GISTs that lacked HLA-A were also negative for HLA-B and -C molecules and thus had a total loss phenotype (Table 3). In addition, half of the tumor samples displayed partial defects of HLA expression in the sense that the staining intensity was decreased or some parts of the tumor were negative. Normal HLA expression was found in only 30% of the tumor samples. Importantly, staining for β2m, the nonpolymorphic light chain of HLA class I revealed that all GISTs expressed this molecule, albeit to varying extent. This indicated that HLA loss was not caused by a deficiency in β2m and suggested that other (nonclassical) HLA molecules might be responsible for surface expression of β2m. Intensity of β2m staining related to the presence of HLA molecules confirming that total loss of HLA indeed leads to lower β2m levels. Overall, this extensive loss of HLA class I molecules in GISTs suggests an active interaction with the immune system resulting in selection of tumor variants with resistance to tumor-directed T cells.

Subdivision of T cells revealed the presence of FoxP3$^+$ regulatory T cells

To determine which types of T cells were comprised in the total CD3$^+$ population, we performed a 3-color fluorescent staining for CD3, CD8 and Foxp3 by which we could distinguish cytotoxic T cells, helper T cells and regulatory T cells. All three T cell subpopulations were detected in GIST samples, as shown for one example in Figure 1*a*. High numbers of infiltrating cytotoxic T cells (CD3$^+$CD8$^+$FoxP3$^-$) and T helper cells (CD3$^+$CD8$^-$FoxP3$^-$) were found in the GIST samples, up to 799 cells/mm^2 for cytotoxic T cells (median 39 ± 146 cells/mm^2) and up to 447 cells/mm^2 for T helper cells (median 71 ± 123 cells/mm^2) (Fig. 1*b*). These high numbers of infiltrated effector T cells supported the notion of active interactions between the immune system and GIST, as suggested by the frequent loss of HLA class I molecules. Regulatory T cells were visualized by cell surface staining of CD3 and compact nuclear staining of FoxP3, as observed with confocal microscopy (Fig. 1*a*). The number of regulatory T cells was much lower than that of the other subtypes (median 8 ± 17 cells/mm^2), but were detectable in nearly all GIST samples (Fig. 1*b*). We recently described that the corresponding counts of CD8$^+$ and FoxP3$^+$ T cells for each tumor were an important and independent prognostic factor for patients with cervical cancer,[28] indicating that the balance between immune activation and inhibition determined the outcome of tumor development. Compared to this cohort, the ratios of cytotoxic T cells *versus* regulatory T cells in the GIST samples (median 3.9 ± 9.5) were more than 6 times lower, indicating a strong immune suppressive environment in GIST. Importantly, the degree of infiltration by these two T cell subpopulations strongly correlated ($R = 0.675$, $p < 0.0001$), so that tumors with high numbers of infiltrating effector T cells also contained high numbers of regulatory T cells. Of note, no statistical significant differences were found for the T cell numbers or ratios between primary and metastatic lesions, or other clinical parameters. We conclude that GIST is heavily infiltrated with all T cell subpopulations, but the low ratios of cytotoxic T cells / regulatory T cells point at an immune suppressive microenvironment in the tumors.

M2 type macrophages characterize the immune infiltrate of GIST

We then analyzed the myeloid lineage in further detail. Immunohistochemical stainings revealed that cells expressing CD1a, DC-LAMP (CD208) and S100 were hardly present in GISTs, but that CD68 positive cells were abundantly present (Table 2). This indicated the dominance of macrophages in the tumor infiltrate and that the number of dendritic cells was negligible. To further substantiate the role of macrophages and to investigate whether these cells were polarized towards an M1 or M2 like differentiation status, we analyzed the immune infiltrate for expression of the scavenger receptor CD163. This receptor is typically displayed on M2 type macrophages[26,33] and staining of the GIST samples revealed an extensive number of cells (Fig. 2*a*). CD163 stainings of 4 different GISTs are displayed in Figure 2*a*, showing the diversity of the tumor samples and illustrating that the presence of macrophages is sometimes overwhelming. Very similar patterns were observed as with the pan-macrophage marker CD68. Images of CD163 stained sections were computer-

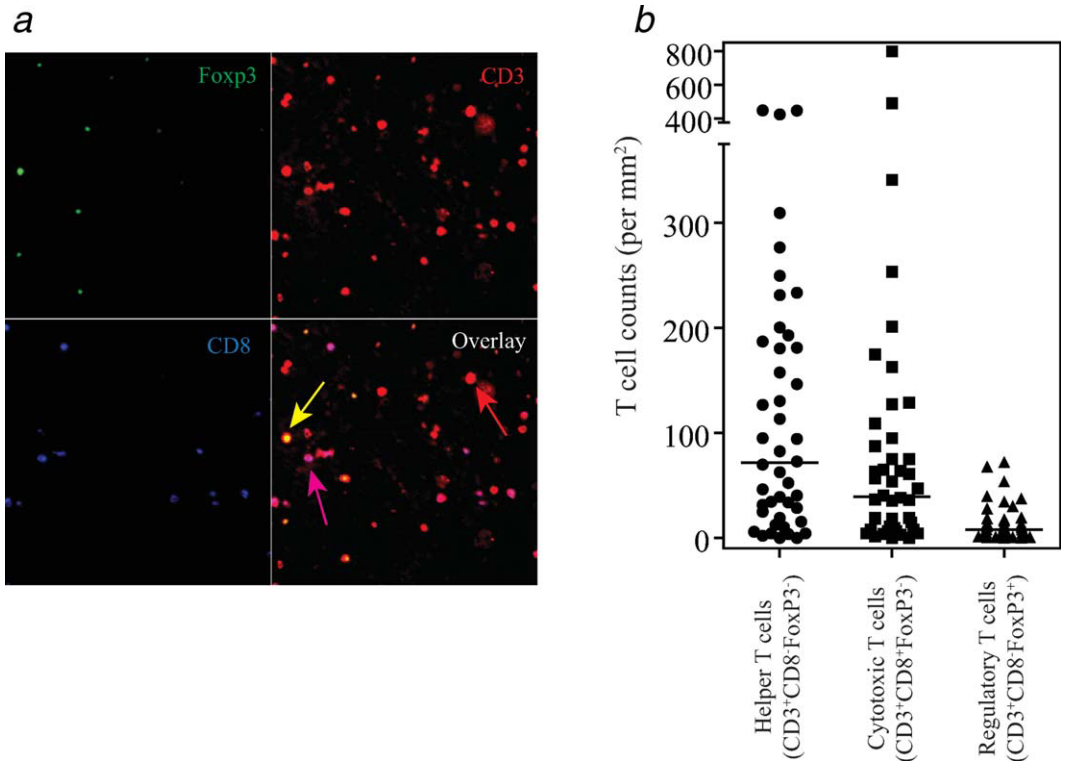


Figure 1. Analysis of T cell subsets in gastrointestinal stromal tumors. Three-color fluorescent stainings were performed on the cohort of GIST samples. (*a*) An example of a tissue section with FoxP3 (green), CD3 (red) and CD8 (blue) and the overlay. Confocal microscopy was used for evaluation to confirm a nuclear FoxP3 staining accompanied with surface CD3 staining in regulatory T cells. Cytotoxic T cells were purple due to the combination of CD3 and CD8. Single CD3 positive cells were considered helper T cells. Arrows in the overlay picture indicate examples of regulatory T cells (yellow), cytotoxic T cells (purple) and helper T cells (red). (*b*) Infiltrating T cells were enumerated for 10 pictures per tissue section and calculated for number of cells per mm^2. Lines in the graph indicate the medians of each type of T cells observed in the tumors.

assisted scored and expressed as a percentage of positive surface area of the tumor sample to obtain an objective value. CD68 and CD163 counts strongly correlated, indicating that M2 type macrophages are dominantly represented in the total pool of macrophages (Fig. 2*b*). In addition, the expression pattern of HLA-DR, which is a HLA class II molecule, was also very comparable to that of CD163, suggesting that the M2 type macrophages are HLA-DR positive. To examine the presence of M1 type macrophages, we performed a double fluorescent staining with CD163 in combination with CD14 (Fig. 2*c*). Overall, the median number of cells expressing CD14 was 79.5 ± 226 per mm^2, so, much lower than that of CD163. The total number of CD163 positive cells was comparable to our previous conventional stainings. Some of the CD163 positive macrophages also displayed CD14 at the cell surface (yellow cells in Fig. 2*c*), but the majority was single positive for CD163. Single CD14 positive macrophages were also detected, suggesting that GISTs are also infiltrated with M1 type macrophages, albeit to much lower degree (Fig. 2*c*). To assess the intratumoral presence of myeloid-derived suppressor cells (MDSC), we performed a triple fluorescent staining with a combination of antibodies against CD14, CD33 and HLA-DR. We did observe CD33 positive cells (median 29.4 ± 96/mm^2), but $CD14^-CD33^+HLA\text{-}DR^-$ cells, described as being MDSC, were absent in GISTs. Nearly all CD33 positive cells co-stained with CD14 and/or HLA-DR. Only in one tumor, which also had high infiltrate of M2 macrophages and T cells, we detected some MDSC. These myeloid markers confirmed our notion that M2 type macrophages are most abundant in GIST.

The number of infiltrating CD163 positive macrophages was then analyzed as a variant of clinical parameters and a significant difference was observed for primary *versus* metastatic tumors. Metastatic lesions contained on average twice the number of macrophages as primary lesions, 4.9% *versus* 2.7% surface area, respectively (Fig. 2*d*), implying that the presence of M2 macrophages is associated with tumor progression. No differences were found for other clinical parameters, such as gender, tumor location, tumor size or tumor mitosis index. Next, a Spearman correlation analysis of the macrophage infiltrate revealed a significant association of macrophages with regulatory T cells (Fig. 2*e*). Interestingly, helper T cells and cytotoxic T cells were not significantly correlated with macrophages, indicative for an anti-

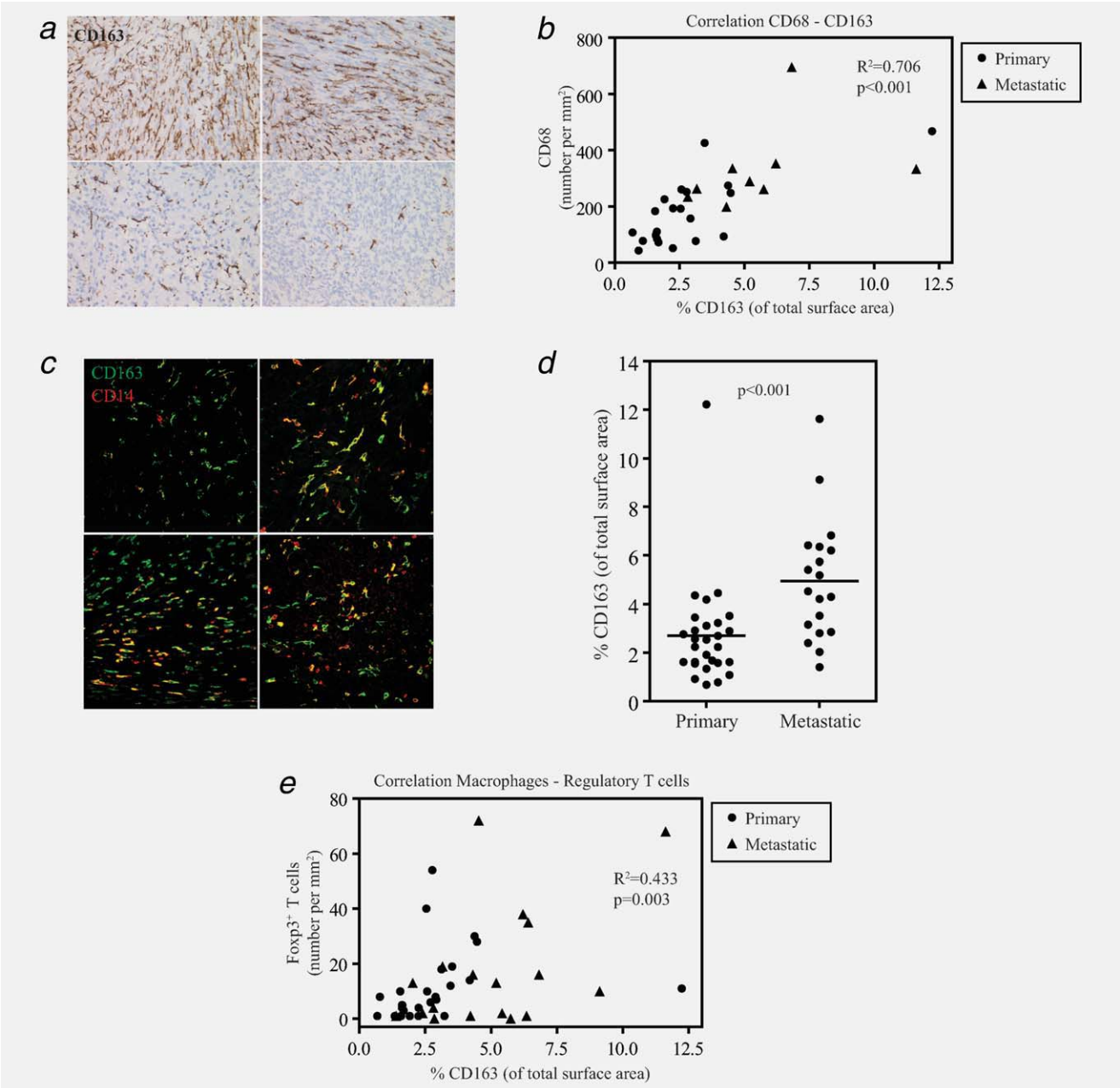


Figure 2. Analysis of infiltrating macrophages in GIST. (*a*) Four examples of CD163 staining, illustrating the diversity of infiltration by macrophages and the extreme degree of M2 type macrophages in some tumors. Pictures of these tissue sections were computer-assisted analyzed and expressed as percentage out of the total surface area of the image. (*b*) Counted CD68 positive cells, representing all macrophages, correlated with percentages of CD163 values, indicating that M2 type macrophages are represented in all GIST samples. Primary and metastatic GISTs were separately depicted with different symbols. Correlation analysis was performed with Spearman rank test. (*c*) Double fluorescent stainings of 4 GIST samples with CD14 (red) and CD163 (green). Double positive cells stain yellow. CD163 positive macrophages are dominantly present in the tumor and part of them co-expresses the monocyte marker CD14. Some CD14 single positive cells can be observed and these might represent M1 like macrophages. (*d*) Counts of CD163 positive cells for primary tumors were compared to those for metastasized tumors. Metastasized GISTs harbored significantly more macrophages than primary GIST (Mann-Whitney analysis). Bars indicate the means of each group. (*e*) Correlation analysis between regulatory T cells and macrophages. Primary and metastasized GIST samples were depicted with different symbols. Correlation analysis was performed with Spearman rank test.

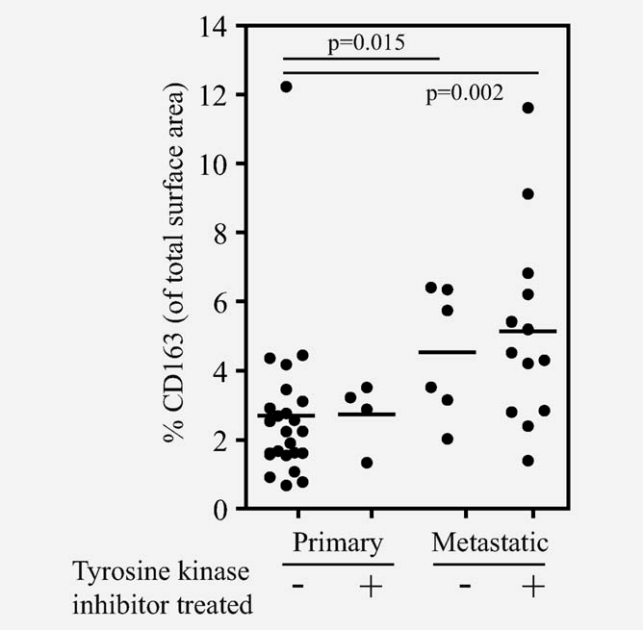


Figure 3. Number of infiltrating macrophages is not influenced by tyrosine kinase inhibitor treatment. The degree of macrophage infiltration in GIST, quantified by CD163 staining, was compared in 4 clinical groups: non-treated primary, treated primary, non-treated metastases and treated metastases. These data indicate that the stronger macrophage infiltration in metastases is related to tumor progression and not to treatment with tyrosine kinase inhibitors imatinib and sunitinib. Groups were significantly different from each other (Kruskal Wallis test, $p = 0.004$). Where significances are not depicted, p values were higher than 0.05. Lines represent the means of each group.

inflammatory role of M2 macrophages. These analyses suggested that macrophages are implicated in the number of regulatory T cells in the local tumor environment and support our previous *in vitro* data demonstrating the generation of regulatory T cells upon encounter with M2 macrophages.[30] So, GISTs and particularly metastases of GISTs are characterized by infiltrating M2 macrophages and regulatory T cells, both anti-inflammatory immune cells, which may affect tumor progression through dampening local cytotoxic immune responses.

Influence of tyrosine kinase inhibitors on infiltrating macrophages

Most of the metastatic GIST patients included in our study had been treated with imatinib and some with subsequent sunitinib administrations. The fact that we observed high numbers of macrophages and regulatory T cells in the metastatic lesions could implicate a role for these therapeutic tyrosine kinase inhibitors. To distinguish between these two factors, intrinsic tumor effect or induction by the inhibitors, we analyzed the CD163 counts in several groups within our GIST cohort. Metastatic tumors from untreated patients contained significantly more infiltrated M2 macrophages compared to primary untreated tumors (Fig. 3), indicating that the abundance of these cells is most likely not the result of the tyrosine kinase inhibitors, but is related to tumor progression *per se*.

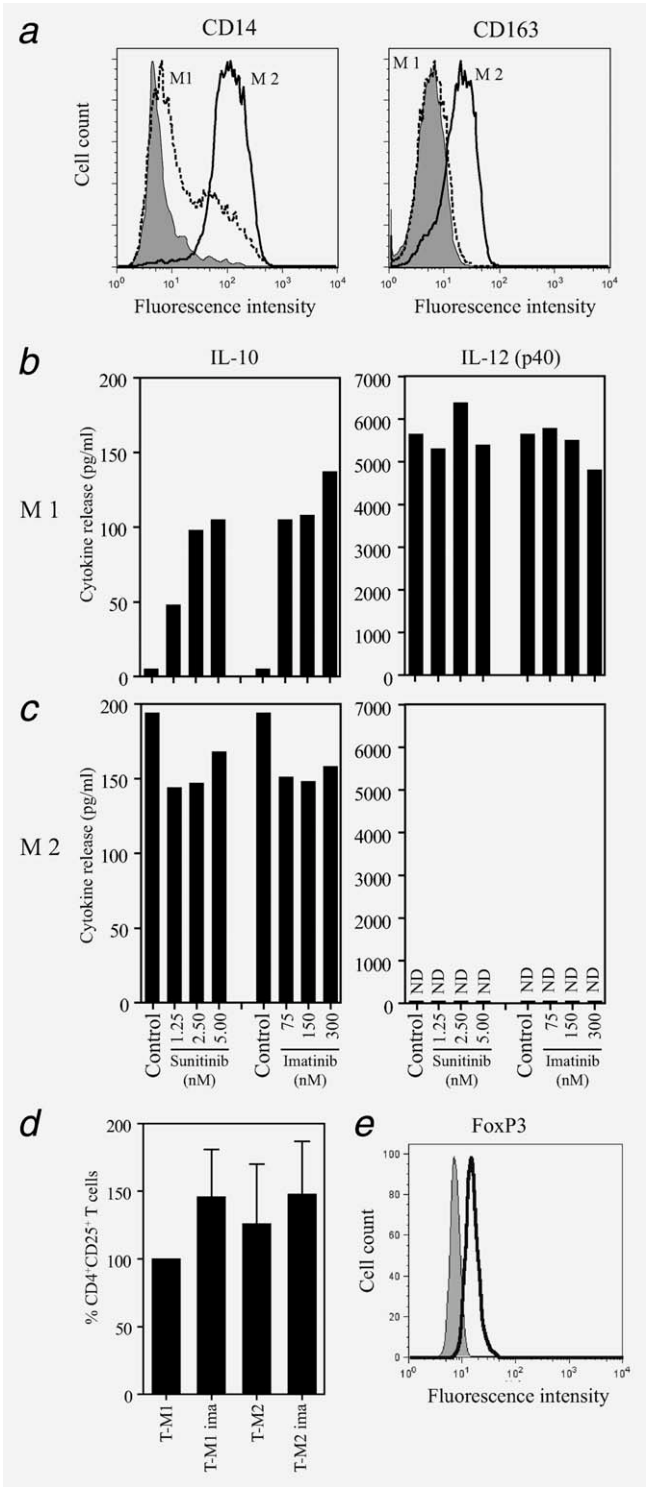
Inflammatory M1 type macrophages produce IL-10 upon incubation with imatinib or sunitinib

Although treatment with the tyrosine kinase inhibitors imatinib and sunitinib was not associated with altered numbers of infiltrating immune cells, we reasoned that these therapeutics could still affect the function of macrophages. We tested this possibility in monocyte cultures that were differentiated into M1 or M2 subtypes.[29,30] M1 macrophages typically displayed a $CD14^+CD163^-$ phenotype and produced high levels of the p40 chain of IL-12 and IL-23, but no IL-10 (Figs. 4*a* and 4*b*), whereas anti-inflammatory M2 macrophages do expressed CD163 and only produced IL-10 (Figs. 4*a* and 4*c*), in line with our previous findings.[29,30] Strikingly, low concentrations of imatinib and sunitinib during the monocyte cultures resulted in production of the anti-inflammatory cytokine IL-10 by M1 type macrophages (Fig. 4*b*). The IL-10 levels were comparable to those produced by M2 type macrophages (Fig. 4*c*). The cytokine profile of M2 macrophages did not change. To assess if the imatinib-treated M1 macrophages were able to induce regulatory T cells *in vitro*, we performed cocultures with PBMC using a protocol we previously developed.[30] Pretreatment of M1 macrophages with imatinib resulted in 45% more $CD4^+CD25^+$ T cells compared with normal M1 cells (Fig. 4*d*). This T cell subset stained positive for FoxP3 (Fig. 4*e*) indicating that imatinib can indeed modulate the capacity of macrophages to induce regulatory T cells. Together, these data indicate that tyrosine kinase inhibitors do not alter the degree of immune infiltrate in GIST, but might skew the function of infiltrating M1 cells towards an anti-inflammatory IL-10 producing macrophage, and that this effect adds to the abundant presence of M2 type macrophages and regulatory T cells to promote immune evasion.

Discussion

We have investigated the immune infiltrate in GIST and found that these sarcomas are heavily infiltrated with macrophages and T cells. Detailed subtyping with unique and specific markers revealed immunosuppressive phenotypes in both lineages. A relative high frequency of T cells expressed the nuclear factor FoxP3, which is functionally determining regulatory T cells and the macrophages displayed the CD163 scavenger receptor, characteristic for anti-inflammatory M2 subtype.[26,33] Importantly, also cytotoxic T cells and helper T cells were detected in high numbers, as well as some M1 type macrophages, resulting in an emerging picture of active involvement and interaction of immune cells in GIST. Our previous work in cervix carcinoma showed that the ratio between cytotoxic T cells and regulatory T cells is an important independent prognostic factor,[28] implying that the

balance of activating and inhibiting factors finally determines the outcome of antitumor immune responses. The important prerequisite to determine this balance is to dissect the total pool of T cells into the different subtypes. We applied a 3-color fluorescent staining to simultaneously enumerate these subtypes in tumor samples and found that the balance in GIST clearly tips to regulatory T cells.



The number of M2 macrophages in the tumor samples correlated with the number of infiltrating regulatory T cells, but not with cytotoxic T cells, suggesting that local anti-inflammatory macrophages might induce regulatory T cells. In our previous studies, we found that M2 type macrophages indeed are capable of generating suppressor T cells in culture systems.[30] The local interplay between GIST cells, macrophages and regulatory T cells in the microenvironment of the tumor is most likely important for the growth and progression of the lesions and for dampening cytotoxic immune effector functions. Interestingly, metastatic GIST lesions harbored twice as much M2 type macrophages as primary lesions. This abundant presence of macrophages in metastatic GIST was not related to treatment with tyrosine kinase inhibitors imatinib and sunitinib, as untreated GIST metastases also contained a high degree of M2 macrophages. Although the number of immune infiltrating cells was not altered by these kinase inhibitors, we found that monocytes are very sensitive for induction of IL-10 secretion under influence of imatinib and sunitinib. Low concentrations of the tyrosine kinase inhibitors resulted in production of this anti-inflammatory cytokine by M1 polarized cells, whereas the normal secretion of IL-12p40 was unchanged. Overall, our study indicates that immune suppressive mechanisms are active in

Figure 4. Cultured M1 type macrophages produce IL-10 upon incubation with tyrosine kinase inhibitors imatinib and sunitinib. Monocyte derived cultures of M1 and M2 type macrophages from healthy PBMC donors were analyzed by flow cytometry and cytokine production. (*a*) Cell surface expression of CD14 and CD163 was examined with flow cytometry. The profiles of M1 and M2 type macrophages, $CD14^+$ *versus* $CD14^+CD163^+$ confirmed our earlier studies[30] and indicated that the tyrosine kinase inhibitors imatinib and sunitinib did not alter the marker phenotype of the cells. (*b*) Cytokine production by M1 type macrophages revealed that low concentrations of both inhibitors induced IL-10 release, but did not alter the production of IL-12 (p40 chain). (*c*) Cytokine production by M2 type macrophages was not altered by the incubation with tyrosine kinase inhibitors. ND = not detected. The used concentrations of imatinib and sunitinib did not result in toxicity of the cells. Cytokine measurements were from pooled triplicates and representative results from 3 independent experiments are shown. No differences were detected between 3 different PBMC donors. (*d*) PBMC were cultured for 2 weeks in the presence of M1 or M2 type macrophages that had been differentiated with or without imatinib ('ima', 300 nM). The frequency of regulatory $CD4^+CD25^+$ T cells in the cultures was determined by flow cytometry. The values are displayed as relative to the frequencies found for M1 type macrophage cultures. The means and standard deviations from 4 independent experiments are depicted. (*e*) Flow cytometry analysis on intracellular FoxP3 expression in the $CD4^+CD25^+$ T cells from the co-cultures with M1 type macrophages that were differentiated in the presence of imatinib. Filled grey histogram represents the isotype control staining.

GIST and that anti-inflammatory M2 type macrophages are a dominant characteristic of metastasized and tyrosine kinase inhibitor-treated GIST.

Interestingly, previous reports pointed at an immune stimulatory role for tyrosine kinase inhibitors.[19,34–38] Imatinib was shown to induce the proinflammatory cytokine IFNγ in NK cells. IFNγ is a well-known cytokine that is important in protective immune responses against tumors. The effect of imatinib on NK cells seemed to be mediated by dendritic cells and was most prominent when administrated together with the activating stimulus Flt-3L, which promoted the crosstalk between dendritic cells and NK cells. Furthermore, patients with GIST that elicited an imatinib-triggered IFNγ response had a longer progression free survival.[36] Recently, sunitinib was reported to reduce the frequency of MDSC in the blood of renal cell carcinoma patients.[19,35] Concomitantly, the number of regulatory T cells also decreased. At first sight, these results seem to contradict the immune suppressive effect of imatinib and sunitinib we found in our study. Multiple groups reported that imatinib strongly inhibits the outgrowth of monocytes from CD34 progenitor cells.[14,16,17,39] This finding on monocyte hematopoiesis can easily be explained by the fact that the M-CSF receptor (cFMS) is silenced by imatinib, as it also belongs to the same type III family of tyrosine kinases as cKIT.[12,14] The immune deviating effects of imatinib, however, might be much more subtle. We found that imatinib promoted the production of IL-10 by M1 macrophages, but the other features of M1 cells were not affected: they still produced IL-12p40 and were negative for CD163. Likewise, some studies have found impaired maturation of dendritic cells from monocytes,[40] but this effect was not overt. In conclusion, the blood concentrations of imatinib and sunitinib in patients might result in subtle changes of the differentiation status of myeloid cells, leading to activation of NK cells at one hand and anti-inflammatory functions in macrophages at the other. In the end, however, the local tumor microenvironment critically determines the traits of growth, spread and immune deviation. In that respect, it is noteworthy that we did not find infiltrating dendritic cells or NK cells in the GIST lesions, even not in imatinib and sunitinib treated samples. Of note, we applied antibodies against the NK-specific receptor NKp46 for the detection of NK cells, which has the major advantage above CD56-directed antibodies that subsets of T cells are not stained.[41] This readily explains the difference with a previous report on GIST infiltrate analysis.[42]

Tumor-associated macrophages (TAM) have been associated with tumor vascularization, metastases and poor prognosis.[26] TAM often display features of M2 type macrophages, which normally play a role in controlling tissue damage and wound healing.[26,27] In leiomyosarcomas and gliomas, the degree of infiltration with M2 type macrophages, as determined by CD163 positive cells, was associated with a worse prognosis,[43,44] a finding that corroborates our study. The exact signal transduction pathways that are responsible for the M2 differentiation of TAM have not been charted yet, although the NKκB involvement is evident.[45] Interestingly, manipulation with these critical master regulators in macrophages had marked consequences for tumor growth,[45] suggesting that changing the polarization status of TAM might be a promising approach for treatment of GIST in the future.

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