Introduction  Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract. They are regarded as having relatively uniform histology, although their potential for malignant behavior varies. Despite a strong promoting role of tumor-infiltrating innate immune cells in neoplastic progression, the presence of immune cells in GISTs has not yet been studied.

Methods  A total of 47 untreated, c-kit-positive primary GISTs were immunohistochemically analyzed to distinguish histiocytic and dendritic cells (DCs) (KIM-1P, fascin, and CD68) from cells of lymphoplasmacellular origin (CD3, CD20, and CD56). Furthermore, the gene expression of proinflammatory cytokines was characterized by real-time, reverse transcription-PCR analysis of total RNA extracted from frozen tissue samples.

Results  KIM-1P+ cells were the dominant immune cells (681 ± 295 cells/mm²) and were scattered among the tumor cells. Most of the KIM-1P+ cells showed cellular projections characteristic of DCs. Fascin positivity identified a subgroup of DCs. In comparison to KIM-1P+ cells, there were significantly fewer CD68+ macrophages (196 ± 217 cells/mm²). CD3+ T cells were the dominant lymphocytes (201 ± 331 cells/mm²), whereas B cells (60 ± 126 cells/mm²) were few. On transcriptional level, a concomitant gene expression of cytokines for the classical acute phase cytokines TNF-α and IL-6 was missing, thus supporting the rather innate status of immune cells.

Conclusion  GISTs contain, beside T lymphocytes, a high number of monocyte-derived cells, which we suggest are, at least in part, immature DCs. Together with the lack of gene expression of inflammatory cytokines in tumor tissue our results point to a possible ‘symbiotic relationship’ between the tumor and the local immune cells. Eur J Gastroenterol Hepatol 20:327–334 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: dendritic cells, gastrointestinal stromal tumor, immune response

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The presence of immunocytes within GISTs has not been analyzed earlier. The aim of this study was to differentiate histiocytic cells and DCs from cells of lympho-cellular origin, and to determine their maturation and ‘activation’ status in 47 c-kit-positive primary GISTs using immunohistochemistry, and for proinflammatory cytokines using real-time, reverse transcription-PCR.

Methods
This study included 47 c-kit-positive primary GISTs. The evaluation of malignancy was performed according to Miettinen et al. [15] and the risk of aggressive behavior was estimated according to Fletcher et al. [16]. Ethics approval for this study was obtained from the Ethics committee of the University of Göttingen.

Immunohistochemistry
For GIST diagnosis, antihuman c-kit, and CD34 protein antibodies were used. The proliferation rate was evaluated using the Ki67 antigen (MIB1) from the areas with the highest mitotic activity. Immunohistochemistry was performed using the alkaline phosphatase method on formalin-fixed and paraffin-embedded tissue sections.

Several markers were used to characterize the different leukocytes. KIM-1P is a fibrohistiocytic marker, which belongs to the mononuclear phagocyte system antigens [17]. It is supposed to detect tissue macrophages [18] and a population of CD1a-negative DCs [17]. CD68 is a general marker for macrophages [19]. To differentiate between macrophages and DCs, the antifascin antibody was used [20]. This antibody detects core actin bundles of dendritic projections [21]. CD83 is a surface antigen present on mature DCs [22], whereas staining of single cells with the S-100 protein is considered as a marker for immature DCs [9]. S-100 protein, however, is also used in the diagnosis of schwannomas [23,24]. CD3 [25] and CD20 [26] differentiate between T cells and B cells, respectively. CD56 is a surface marker of natural killer (NK) cells [27]. The optimal working dilutions of the antibodies used in this study are listed in Table 1.

As a negative control, nonimmune serum was used. For antigen retrieval, the slides were cooked in citrate buffer (10 mmol/l, pH 6) for 45 min in a steam cooker or pretreated with protease (Sigma, Steinheim, Germany) for 10 min at 40°C. After cooling, the slides were washed in distilled water, followed by a washing step with Tris-buffered saline (TBS; 7.4% without Tween) for 5 min. They were then incubated in TBS (without Tween) with bovine serum albumin (2%) for 10 min. After removal of bovine serum albumin/TBS, the specimens were incubated with the primary antibody for 30 min. For visualization of antigen–antibody binding, the ChemMate Detection kit (K5005, DAKO, Glostrup, Denmark) was used. Double-immunofluorescent staining was done for some cases with the c-kit and antifascin antibodies, for which it was technically feasible. The primary antibodies were used at the same concentration as for conventional immunohistochemistry. They were visualized with anti-rabbit alexa 555 (red) and antimouse alexa 488 (green) (Molecular Probes, Invitrogen). Cell nuclear counterstaining was done with 4,6-diamidino-2-phenylindole (Molecular Probes, Invitrogen) for 20 min. Immunohistochemical staining for cells of the immune system was evaluated in a total of 35 cases for KIM-1P, CD68, and CD3; in 27 cases for S-100; and in a subgroup of 14 cases for CD20, CD56, CD83, and fascin.

Image analysis
For the count of antigen-stained cells, two representative photographs per slide of the tumor tissue were taken, at a magnification of 80×. The resulting image size was 1320 × 990 μm (1.3 mm²) for this magnification. For further analysis, we have written a small set of IDL programs that result in a black and white image, with the stained regions being white and the unstained regions black. Inbuilt IDL routines then numbered the distinct white structures. Structures smaller than 10 pixels were discarded and structures greater than 50 pixels were checked for substructure using a new threshold. The final match between the results of the computer analysis and the actual staining was confirmed by superimposing the outlines of the white regions on the original image. The correctness of the results of this computed analysis was validated visually.

Quantitative realtime, reverse transcription-PCR
RNA was extracted from snap-frozen tissue samples, according to the trizol method described earlier [28]. Reverse transcription was done using moloney murine leukaemia virus reverse transcriptase (Invitrogen,

| Antibody | Dilution | Pretreatment antigen retrieval (citrate) | Vendor (Cat. no.) |
|----------|----------|----------------------------------------|-------------------|
| c-kit (CD117) | 1 : 200 | Heat | DAKO (A4502) |
| Aktin | 1 : 50 | None | Immunotech (PN IM 1144) |
| Desmin | 1 : 1 | None | Immunotech (IM 1073) |
| S-100 | 1 : 20 | Protease | Immunotech (IM 1071) |
| CD34 | 1 : 20 | None | Immunotech (Cat. no. 1185) |
| MIB1 (K67 clone MIB1) | 1 : 200 | Heat | DAKO (M7240) |
| CD68 (clone KP1) | 1 : 6000 | Heat | DAKO (M0814) |
| KIM-1P | 1 : 6000 | Protease | Pathology, Kiel* [18] |
| CD83 (clone 1H4b) | 1 : 50 | Heat | Novo Castra (NCL-CD83) |
| Fascin (clone 55K2) | 1 : 1000 | Heat | Chemicon International (MAB3582) |
| CD3 (polyclonal) | 1 : 100 | Heat | DAKO (A0452) |
| CD20cy (clone L26) | 1 : 200 | Heat | DAKO (M0756) |
| CD56 (clone 123C3) | 1 : 100 | Heat | Zymed (18-0152) |

*Commercially not available, donation by Professor Parwaresch, Kiel.
Sequences of gene-specific primers used for quantitative real-time, reverse transcription-PCR analysis

| Gene          | Primer sequence          | Product size (bp) |
|---------------|--------------------------|------------------|
| IL-4 (tv 1)   | Sense: 5'-CCG ACA GAAG TGT GGA CAT TAT | 148              |
|               | Antisense: 5'-CAT TGC GAC AAG TTT CCA TAG |                |
| IL-6 (IFN-β2) | Sense: 5'-CCG ACA GAAG TGT GGA CAT TAT | 148              |
|               | Antisense: 5'-CAT TGC GAC AAG TTT CCA TAG |                |
| IL-10         | Sense: 5'-GAG GGG ACC GCA CAG AAT CAT | 117              |
|               | Antisense: 5'-GAG GGG ACC GCA CAG AAT CAT |                |
| IL-12p        | Sense: 5'-GAC CAA TCA TCA AAT TGT GGA CAT | 117              |
|               | Antisense: 5'-GAC CAA TCA TCA AAT TGT GGA CAT |                |
| TNF-α         | Sense: 5'-GAC CAA TCA TCA AAT TGT GGA CAT | 117              |
|               | Antisense: 5'-GAC CAA TCA TCA AAT TGT GGA CAT |                |

G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, IFN-gamma-inducible protein 10; TNF, tumor necrosis factor.

Statistical analysis
Values for the count of immune cells are expressed as mean ± SD. Significant differences between the means were evaluated using analysis of variance, followed by Student’s t-test. Differences of P values less than 0.01 were considered to be statistically significant. The summary of the PCR data is shown as a box-and-whisker plot. These data were analyzed using the Mann–Whitney U-test between two unpaired groups, with P values less than 0.01 considered to be significant.

Results
Histopathological findings
Of the 47 primary GISTs, 29 tumors (62%) were localized in the stomach, 15 (32%) in the small intestine, and three (6%) in the large intestine. The mean age of the patients at the time of tumor resection was 66 years (age range: 35–86). In all, 19% of the cases were classified [15] as benign, 30% as low malignant, and 51% as malignant. The risk for aggressive behavior [16] was estimated to be very low in 4.3%, low in 25.5%, intermediate in 27.6%, and high in 42.5%.

In all, 75.5% of the tumors showed a spindle cell phenotype, 6.5% an epithelioid phenotype, and 17.5% a mixed phenotype. Aside from immunohistochemical c-kit positivity (100%), CD34 positivity was detected in 40/47 (85%) tumors. No fibrotic reaction was observed within the tumor.

Immunohistochemical characterization of immunocytes
The general distribution of the various immune cells was diffuse, with the immunocytes being scattered between the tumor cells, independent of the tumor location within the gut. The number of immune cells within this pattern was analyzed quantitatively. To a small extent, immune cells further colocalized as aggregates in regressive tumor areas.

Evaluation of immature and mature dendritic cells
Figure 1 shows consecutive tumor sections of a representative GIST. For comparison, Fig. 1a shows the corresponding hematoxylin and cosin staining. KIM-1P + cells had cell surface projections typical for DCs (Fig. 1b). A large number of spindle-cellular KIM-1P + cells were scattered between the tumor cells and along tumor cell bundles. In regressive tumor areas, they showed round morphology. KIM-1P + cells were significantly more abundant (mean: 85 ± 295 cells/mm², median: 855 cells) than CD68 + cells (mean: 196 ± 217 cells/mm², median: 125 cells; P = 5 × 10⁻¹⁶). The microscopic appearance of CD68 + cells varied from round cells to cells with DC-like appearance (Fig. 1c). CD68 + cells with spikes were located between the tumor cells. In regressive areas of the tumors and within lymphocellular aggregates, CD68 + cells were round and...
larger, with strong CD68 positivity as in lysosomal rich macrophages.

To further discriminate DCs from macrophages, the antifascin antibody was used. Fascin + cells with their typical dendritic projections were located between the tumor cells. The antifascin antibody also stained endothelial cells and in some cases the tumor cells. To differentiate fascin + DCs from tumor cells, double fluorescent staining was performed using both the antifascin antibody and the anti-ckit antibody (Fig. 2). Even though there were a good number of fascin + cells scattered between the tumor cells, we could not detect mature DCs with CD83 positivity. We, therefore, analyzed the cytomorphology of S-100-positive cells in GISTs, which is known as a marker for immature DCs [9]. S-100 + cells showed small cell surface spikes and projections, typical for DCs in the antigen-collecting mode (Fig. 3). In comparison to the number of KIM-1P + cells, there were only a few S-100 + cells (mean: 40 ± 50 cells/mm², median: 26 cells). In all, 57% of the tumors showed single S-100-positive cells scattered within the tumor cells, whereas only one of 47 (2%) of the tumors showed focal S-100 positivity of the tumor cells.

**Evaluation of lymphocytes**

Evaluation of the lymphocytic infiltrate showed that CD3 + T cells were the most dominant lymphocytes within GISTs (Fig. 4a). In tumors with an abundant lymphocytic infiltrate, CD3 + cells were scattered between the tumor cells, otherwise they were preferentially located perivascularly and in regressive tumor areas. The mean cellular count was 201 (± 331) CD3 + T cells per mm². Comparatively few CD20 + B cells (mean: 60 ± 126 cells/mm²) could be detected (Fig. 4b). They were mainly located around the blood vessels of the tumor. An even smaller number of CD56 + NK cells were present (Fig. 4c). They were detected as small
round CD56 + cells scattered within the tumor tissue (34 ± 41 cells/mm²).

**Association with clinicopathological parameters**

The various immunocyte markers KIM-1P, CD68, S-100, CD3, CD20, and CD56 showed no association with tumor size, location, phenotypic appearance of tumor cells, or prospective biological behavior. Only the number of CD68 + cells increased significantly from low to high malignancy (P < 0.01; Fig. 5).

**RNA expression of cytokines**

**Classical acute phase mediators and interferons**

In GISTs, specific transcripts for the acute phase cytokine IL-6 were detectable, but low (CT value: 30 ± 2.7). The TNF-α expression was lower, with no detection in 12 of 34 cases (and a mean CT value of 33 ± 1.7 in the remaining cases). The corresponding levels of TNF-α expression (see Methods for the formula) were significantly lower, on average 32 times than those of IL-6 (Fig. 6). IL-6 expression, however, was six times lower than IL-1β expression (mean CT value of 27.8 ± 2). No significant differences in the expression of these cytokines in terms of tumor location or size were found.

Like the classical acute phase cytokines, IFN-α and IFN-β can be produced by mononuclear phagocytes and can mediate early innate immune responses. IFN-γ is involved in antigen processing at later stages of immune responses and is known to be produced by T lymphocytes and NK cells [13]. In GISTs, IFN-α showed the highest mean expression among the different interferons. IFN-α expression was four times higher than the IL-6 expression. Neither difference became statistically significant.

No correlation was observed between the count of T lymphocytes and IFN expression, and no significant difference of IFN expression with tumor location or size was observed.

The expression of IFN-γ-inducible protein 10, an IFN-inducible chemokine acting on T cell recruitment [30], was 1.8 times higher than IL-6 and 1.5 times higher than IFN-γ (Fig. 6).
Expression of the classic T cell response-relevant cytokines IL-2, IL-4, and IL-10 and of further cytokines involved in ongoing inflammatory reactions, including granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-12α, IL-12β, and IL-15, were not detectable.

**Discussion**

To our knowledge, this is the first study to analyze the dominating immune cells and a concomitant cytokine expression in untreated GISTs. The most abundant immune cells belonged to the mononuclear dendritic phagocyte system. Lymphocytes were also present, however, they were fewer in number. Of these most were T cells. Gene expression of inflammatory cytokines was low or totally absent within the GIST tissue.

Monocytes can differentiate into tumor-associated macrophages (TAMs) or immature DCs [31]. Tumor-infiltrating DCs are expected to capture and process antigens shed by adjacent tumor cells and then migrate to draining lymph nodes [32]. On one hand, tumor-infiltrating DCs have been associated with a better prognosis [33], and it has been speculated that DCs and lymphocytes at the tumor margin prevent tumor growth. For example, DCs were detected in peritumoral areas of rectal carcinoma, forming contacts with lymphocytes along the basement membrane and tumor cells [11]. On the other hand, the question was recently raised whether DC maturation was intratumorously prevented, thus decreasing the efficacy of tumor-associated DCs in controlling tumor growth [5,34]. In breast adenocarcinoma, immature DCs were shown to be interspersed in the tumor mass, whereas mature DCs were confined to the peritumoral area [35]. Our results in GISTs demonstrate that subpopulations of DCs or fibrohistiocytes (KIM-1P+, fascin+, S-100+ cells) are present within the tumor and that these cells are likely to be antigen collecting rather than antigen presenting. We propose that the single S-100+ non-tumorous cells that are seen in GISTs represent a fraction of immature DCs.

In comparison to the abundant number of KIM-1P+ monocyctic cells, the low number of lymphocytes, as well as the low transcription of proinflammatory cytokines within the tumor tissue, support the hypothesis of a rather immature, ‘nonactivated’ state of immune cells within GISTs. The number of CD3+ cells was significantly lower than that of KIM-1P+ cells, and the numbers of B cells, as well as of NK cells, were almost negligible. Our results may support the assumption of a defective maturation of tumor-associated DCs resulting in the lack of T’cell priming, as described for other tumor entities [35,36]. For example, in melanomas, the predominance of immature dermal DCs along with naïve CD3+ T cells, and only scattered CD20+B lymphocytes was observed; occasional CD56+ NK cells were also observed [36].

Similar to DCs, instead of promoting inflammation, TAMs seem to be better adapted for scavenging debris, promoting angiogenesis, and tissue remodeling [37,38]. A link between TAMs in the different malignancies and prognosis has been summarized by Bingle *et al.* [6]. The number of CD68+ macrophages has been described to be variable in peritumoral and intratumoral areas [8]. GISTs are again special in this regard, as they do not show invasive growth, leaving the overlaying visceral fascia...
intact. We thus focused our studies on intratumoral immune cells. For most of the previously studied tumor types (breast cancer [39,40], cervix cancer [41], or transitional cell carcinoma of the urinary bladder [42]), macrophage infiltration has been linked with a poor prognosis. The results of our studies are in agreement with these data, as CD68+ cells were the only immune cells that were significantly increased in malignant compared with low-malignant GIST. The number of the other immunocytes could not be related with tumor dignity or risk of aggressive behavior, as evaluated according to Miettinen et al. [15] and Fletcher et al. [16].

Although we found a large number of immune cells within the tumor tissue, the gene expression of cytokines was low or totally absent. The macrophage-derived cytokines IL-6, TNF-α, IL-1β, and IFN-γ have a pivotal role in induction and amplification of inflammation. Whilst IL-6 stimulates T cell and B cell proliferation and differentiation [43], TNF-α is responsible for neutrophil accumulation [44] and also for IFN-γ production [45]. Similar to IL-1β, it also has angiogenetic properties [46]. In GISTs, RNA expression of the acute phase cytokines was very low, especially for TNF-α, but also for IL-6. IL-1β expression, was six times higher than RNA expression of IL-6. This is in accordance with data from other publications in which IL-1β has been shown to be expressed at tumor sites where it may affect the process of carcinogenesis, tumor growth, and invasiveness [47,48].

Cytokines that are derived from activated T cells, such as IL-2 and IL-4, and also the macrophage-derived cytokine IL-10 were at the lower limit of detection in most GISTs. Hence, we suggest that the immature phenotype of local immunocytes does not result from the presence of immunosuppressive molecules such as IL-4 [49] and IL-10 [50], as we could not find transcripts for these cytokines.

Similarly, the mRNA expression of cytokines that are responsible for modulating adaptive immune responses was below the detection level.

IFN transcripts within tumor tissue are less known. Generally IFN-α and IFN-β can be produced by mononuclear phagocytes and mediate early innate immune responses [51]. IFN-γ is involved in antigen processing at later stages of immune responses and is known to be released by T lymphocytes, NK cells, and mature DCs [13]. As for tumors, IFN-γ has been described to be released from activated lymphocytes and NK cells to drive antitumor responses [52]. We could show that IFNs are expressed at the transcript level. We could not, however, find a clear correlation between IFN expression and the studied immunocyte populations.

From our experiments, we conclude that GIST-associated immunocytes with a typical diffuse infiltration pattern might lack activation. This is supported by the presence of immature DCs described in other tumor entities [35,36] and the known low expression of proinflammatory cytokines by TAMs [37]. Our PCR data support these observations. We propose that the lack of inflammation suggests a ‘symbiotic’ relationship between tumor cells and local, nonactivated immune cells in GISTs.

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Conflict of interest: none declared.

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