Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors.

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Abstract

Cancer immunosurveillance relies on effector/memory tumor infiltrating CD8+T cells with a Th1 profile. Evidence for an NK cell-based control of human malignancies is still largely missing. The KIT tyrosine kinase inhibitor imatinib mesylate (IM) markedly prolongs the survival of patients with gastrointestinal stromal tumors (GIST) by direct effects on tumor cells, as well as by indirect immunostimulatory effects on T and NK cells. Here, we investigated the prognostic value of tumor-infiltrating lymphocytes expressing CD3, Foxp3 or NKp46 (NCR1) in a cohort of patients with localized GIST. We found that CD3+ TIL were highly activated in GIST and were especially enriched in areas of the tumor that conserve class I MHC expression in spite of IM treatment. High densities of CD3+ TIL predicted progression-free survival (PFS) in multivariate analyses. Moreover, GIST were infiltrated by a homogeneous subset of cytokine secreting -CD56bright (NCAM1) NK cells that accumulated in tumor foci after IM treatment. The density of the NK infiltrate independently predicted PFS and added prognostic information to the Miettinen score, as well as to the KIT mutational status. NK and T lymphocytes preferentially distributed to distinct areas of tumor sections and probably contributed independently to GIST immunosurveillance. These findings encourage the prospective validation of immune biomarkers for optimal risk stratification of GIST patients.
Introduction

Gastrointestinal stromal tumors (GIST), the most frequent mesenchymal tumor of the digestive tract (10-20 annual cases/million), has become a paradigm for the development of personalized therapies against cancer(1) (2). Since 2001, GIST has served as a role model in the burgeoning field of molecularly defined therapies for solid malignancies. The combined discoveries of a biomarker, an oncogenic gain-of-function kinase mutation(3) and of a highly specific tyrosine kinase inhibitor (4), transformed the prognosis of this disease (5, 6).

Indeed, 70-80% of GIST harbor an oncogenic mutation in the type III receptor tyrosine kinase KIT, leading to ligand-independent receptor homodimerization and consequent kinase activation. The most common mutations of KIT (in two thirds of cases) are in-frame deletions, insertions, substitutions or combinations thereof, affecting the juxtamembrane domain encoded by exon 11. In particular, deletions involving codon 557/558 are associated with a shorter event-free and overall survival (7). Among the GIST bearing wild type KIT, 5-8% exhibit activating mutations of PDGFRA affecting its juxtamembrane domain (encoded by exon 12), the ATP-binding domain (encoded by exon 14) or the activation loop (encoded by exon 18) (8). GIST lacking either KIT or PDGFRA mutations called “wild type GIST” are heterogeneous and harbor mutations in NF1, BRAF, RAS or genes encoding subunits of the succinate dehydrogenase complex (9).

The tyrosine kinase inhibitor imatinib mesylate (IM) was first administered to a GIST-bearing patient in 2001 (5). IM inhibits KIT by directly binding to the ATP-binding site
within the N-terminal lobe of the kinase, stabilizing the kinase in its inactive conformation (10). To date, the median survival of patients with advanced GIST treated with first line IM is 5 years and 34% of the patients survive more than 9 years (11, 12). In addition, adjuvant IM decreases the risk of relapse after resection of a localized GIST (6). However, even long-term IM treatment fails to eradicate GIST cells in both advanced and localized adjuvant settings, perhaps because IM has mostly cytostatic effects or because GIST stem cells do not require KIT signaling for their survival (13). Moreover, continuous IM exposure can lead to the selection IM-resistant tumor cells with secondary KIT mutations (14). An important research goal is to develop new inhibitors interfering with secondary activation loop mutations or targeting new metabolic pathways relevant for distinct subtypes of GIST (such as SDH-deficient tumors).

Another potential strategy consists in mobilizing the immune system against GIST during/after IM therapy. Indeed, immunostimulatory bystander or off-target effects of distinct cytotoxic compounds reportedly contribute to their anticancer effects (15). We found that IM can promote a KIT-dependent crosstalk between dendritic and NK cells, leading to IFNγ production by NK lymphocytes, both in mice and in humans (16). Importantly, in advanced GIST, NK cell activation following 2 months of IM treatment constituted a favorable independent predictive factor (17). Moreover, alternative splicing of exon 4 of the Nkp30 gene affecting the signaling of the NK-specific Nkp30 protein determined the prognosis of metastatic GIST patients treated with IM (18). Beyond NK cells, T cells may influence the course of GIST. Balachandran et al. reported the discovery that, in mouse GIST models, the antitumor effects of IM relied on the contribution of CD8+ T lymphocytes (19). Thus, inhibition of oncogenic KIT by IM in tumor cells could shut down
the expression of indoleamine 2, 3-dioxygenase (IDO), thereby promoting the apoptosis of regulatory Foxp3+ T cells and permitting the activation of tumor infiltrating effector lymphocytes (19).

Very few studies have addressed the immune infiltrate of GIST (20, 21). Using systematic immunohistochemistry (IHC) and flow cytometric analyses, we discovered that primary GIST are infiltrated by activated NK cells and CD3+ T cells. However, the two lymphocytes subsets were not localized in the same tumor areas and influenced progression free-survival independently from currently used prognostic parameters (Miettinen score or KIT mutations) in multivariate analyses.

Results

NK cell infiltrates in GIST

We performed a retrospective immunohistochemical analysis of a cohort of 57 localized and 10 metastatic GIST at diagnosis (IM-free, Supplemental Table 1) using antibodies directed against CD3, Foxp3 and NKp46 aimed at identifying T lymphocytes, regulatory T (Treg) cells and NK cells respectively. The NKp46 specific staining resided in fibrous trabeculae (Fig. 1A, left panel, Fig. 1B), while CD3 and Foxp3 positive cells were mostly localized in tumor foci (Fig. 1A, middle and right panels). The primary tumor of metastatic GIST exhibited similar patterns of tumor infiltrating lymphocytes (TILs) (Fig. 1A). There was no significant correlation between the numbers of T, Tregs and NK cells (Fig. 1C, Fig. 1D). In the absence of IM therapy, harboring a KIT exon 11 mutation or a high Miettinen
score is associated with dismal prognosis (22). In localized GIST, NK TILs were three times less frequent in GIST bearing a KIT exon 11 mutation than in wild type (WT) GIST (Fig. 1E, left panel, median of 6 versus 21/field respectively, p=0.06). In addition, localized GIST with low/intermediate Miettinen risk were enriched in NKp46⁺ (but not CD3⁺) cells and lacked Foxp3⁺ cells at diagnosis compared with high Miettinen risk GIST (Fig. 1F, left panel, p=0.04, and right panel, p=0.01, respectively). When dissecting the 3-digit Miettinen score, we identified NKp46⁺ cells to be significantly associated with a low mitotic index and a stomach localization (Supplemental Fig. 1A-B, left panels, p=0.04 and p=0.01 respectively), while the frequency of CD3⁺ cells inversely correlated with tumor size (Supplemental Fig. 1C, middle and right panels).

To investigate the functional phenotype of NK TILs, we performed a comprehensive flow cytometry analysis of NK cells purified from 14 freshly harvested and dissociated localized GIST tumors (9 at diagnosis and 5 post-IM), comparing them to autologous circulating NK cells or to NK cells purified from 6 soft tissue sarcomas (STS) (Supplemental Table 2). GIST contained denser leukocytic infiltrates than STS (20%±6% of live cells versus 7%±4%) with a marked enrichment in NK cells in the tumor compared with blood (Fig. 2A). Interestingly, in contrast to T cell-edited cancers, GIST did not promote the intratumoral trafficking of CD8⁺ T lymphocytes since most TILs were CD4⁺ T cells (Fig. 2A, Supplemental Fig. 2). The proportion of CD3⁺CD4⁺CD25highCD127⁻ regulatory T cells was not enhanced in TILs compared with blood (Fig. 2A, Supplemental Fig. 2). GIST NK TILs expressed the activation marker CD69 ex vivo (Fig. 2B) and produced IFNγ (and to a lesser extent TNFα) after a brief (2 hours) restimulation with PMA/ionomycine (Fig. 2C). CD3⁺TILs also produced Th1 cytokines in similar conditions (Fig. 2D-E). The phenotype of NK TILs in GIST
was homogeneous with little interindividual or intraindividual (pre- versus post-IM) variations since most GIST NK TIL were CD3^{-}CD56^{bright}CD94^{-}NKp80^{-}CD16^{-}NKG2A^{-}CD158^{-} (Fig. 2F-G) (but expressed variable levels of NKp30/CXCR3/CX3CR1) cells (Fig. 2G). In STS, NK TILs were not only rare but also CD3^{-}CD56^{dim}CD16^{+} and low in the expression of CD94 and NKp80 (Fig. 2G).

Altogether, GIST differ from other sarcomas in thus far that their TIL are enriched in activated CD56^{bright} NK cells that are devoid of inhibitory receptors and are localized at the tumor borders. Moreover, GIST TILs contained activated Th1 cells invading tumor foci.

**Imatinib therapy is associated with MHC class I loss variants and NK cell penetration into tumor foci**

We next analyzed the effects of IM therapy on GIST TIL. Patients received 400 mg IM daily for an average time of 10.7 months. While the density of NKp46 infiltrates did not change in fibrous trabeculae (Fig. 3A-B), the frequency of NKp46^{+} cells infiltrating the core of localized or metastatic GIST increased after IM treatment (Fig. 3B, right panel, p=0.004 for localized and p=0.001 for metastatic GIST). In contrast, Foxp3^{+} cells significantly decreased while CD3^{+} cell numbers did not change (Fig. 3C-D). Hence, the ratio NKp46/Foxp3 in the tumor nests markedly rose by four fold after IM.

Since both T and NK cell education and activation are dictated by MHC class I molecules, we performed immunohistochemical analyses of a total of 42 localized GIST (surgery alone or followed by adjuvant IM, n=32; neoadjuvant IM followed by surgery, n=10) using the EMR8.5 antibody specific for a non-polymorphic epitope of the heavy chain of MHC class I molecules. While most GIST at diagnosis homogeneously express MHC class I molecules, up
to 30% GIST fully lost MHC class I expression upon IM and 40% GIST became partially (in areas) MHC class I negative (Fig. 4A, p=0.008). Importantly, T cells tended to accumulate in MHC class I positive tumors (Fig. 4B, left panel, p=0.03) and areas (Fig. 4C), but disappeared from MHC class I negative tumors or areas (Fig. 4B, left panel, Fig. 4C). In contrast, NK cells were not enriched in MHC class I negative tumors or areas (Fig. 4B, right panel). The immunohistochemical results were corroborated by flow cytometry gating on CD45-KIT+CD34+ cells in 9 GIST at diagnosis and 5 GIST after in vivo exposure to IM. The level of expression of MHC class I molecules was higher in GIST at diagnosis compared with GIST treated with neoadjuvant IM (mean ratio MFI staining/MFI isotype control: 11±4 versus 2±1 respectively, Fig. 4D, p=0.042) while no significant difference was observed for MHC class II, HLA-E or MICA/B expression (Fig. 4D). When analyzing the clinical outcome of the 10 neoadjuvant GIST patients enrolled in this study, we found MHC class I loss variants in all (4/4) patients that were disease-free at 7 years post-diagnosis, while only half of the patients (3/6) relapsing or dying of their disease manifested loss of MHC class I expression.

The reduction of MHC class I expression by GIST tumors did not result from direct oncogene inhibition by IM. We took advantage of IM-sensitive (TF417 cells bearing an exon 11 Kit mutation) and IM-resistant (TF816 cells with an exon 17 kit mutation) KIT transduced erythroleukemic cells (23) that we incubated in the presence of IM and allogeneic NK cells (from normal volunteers). IM significantly decreased cell surface expression of MICA/B but did not affect MHC class I or B7-H6 expression (Fig. 4E). No correlation between the susceptibility of the target to IM and the NK cell effector functions could be observed (Fig. 4F).
We conclude that long-term treatment with IM is associated with a progressive loss of MHC class I molecules on GIST cells, as well as with an increase of NK cells infiltration into the core of the tumor.

**NK cell infiltrates and prognosis of localized GIST**

We analyzed the prognostic impact of the NKp46+, CD3+ and Foxp3+ cell counts in tissue sections at diagnosis (using the median scores defined in Fig. 1) of 53 localized GIST that were treated (n=17) or not (n=36) with IM in adjuvant setting. NK and CD3 cell infiltrates were both associated with a reduced relapse rate (Fig. 5A-B, p=0.001 and p=0.007 respectively) while the Foxp3 score did not influence GIST patients’ clinical outcome (Fig. 5C). Multivariate analyses (with a median follow-up of 51 months) of all relevant clinical parameters available revealed that the Miettinen score, the frequency of CD3 cells (Hazard Ratio=2.26, p=0.05) and the NKp46 scores (Hazard Ratio=0.2, p=0.01) were independent prognostic factors for progression-free survival (PFS) (Table 1). Neither gender nor the mutational status of KIT did significantly influence PFS. Combining NK and T cell scores allowed for the identification of a subgroup of 24% of patients that were NKp46<sub>low</sub>CD3<sub>low</sub> and had a dismal prognosis (Fig. 5D, p=0.0005).

Stratification of GIST patients based on the oncogenic KIT mutation and the NKp46 score identified a subset of 10% of patients that were KIT<sup>wt</sup>NKp46<sub>low</sub> (33% of all KIT WT GIST) with a particularly high relapse rate regardless of IM adjuvant therapy (Fig. 6A p=0.006). Moreover, stratification of GIST patients based on the oncogenic KIT mutation and the CD3 score identified a subset of 33% of patients that were KIT<sup>exon11</sup>CD3<sub>low</sub> (44% of all exon 11 mutated GIST) with a particularly high relapse rate regardless of IM adjuvant therapy (Fig.
6B, $p=0.03$). As expected (22), the Miettinen score alone has an impact on survival with a 5 year median of PFS for patients bearing a high score, while PFS was unreached for GIST patients with Miettinen $^{\text{low/intermediate}}$ score (Fig. 6C, $p=0.006$). Combining both Miettinen and NKp46 parameters identified a subgroup of patients (about one half of Miettinen$^{\text{high}}$ GIST patients) presenting with a Miettinen$^{\text{high}}$ NKp46$^{\text{high}}$ score and a reduced relapse rate, similar to patients with Miettinen$^{\text{low/intermediate}}$ scores (Fig. 6D, $p<0.0001$), regardless of IM-adjuvant therapy (given that 7/11 patients underwent surgery only). In contrast, GIST patients with a Miettinen$^{\text{high}}$ and NKp46$^{\text{low}}$ score exhibited a short PFS (0.91 year versus 5 years for high Miettinen score alone, Fig. 6D), independently of adjuvant-IM therapy (given that 53% of Miettinen$^{\text{high}}$ NKp46$^{\text{low}}$ underwent surgery followed by adjuvant IM therapy). The CD3 score also affected the fate of high Miettinen$^{\text{high}}$ GIST in thus far that a subset of 22% GIST (harboring a Miettinen$^{\text{high}}$ CD3$^{\text{low}}$ score) had a dismal prognosis (Fig. 6E, $p<0.0001$). Thus, in localized GIST, both the CD3$^{+}$ and NKp46$^{+}$ infiltrates can be considered as independent prognostic factors that add relevant information to the classical Miettinen score with regard to the PFS. Overall survival was also analyzed (Table 1). Given the limited follow up and the size of the cohort, no significant correlation with immune infiltrates was observed.

**Discussion**

This report represents, to our knowledge, the first description of a tumor type that appears to be controlled by a natural immunosurveillance system that involved both T and NK cells. We found that imatinib promotes the reduction of MHC class I expression by tumor cells (which theoretically may reflect a T cell-based immunoediting process) and a relocation of
NK cells to the core of the tumor. Finally, NK and T cell infiltrates were both independently associated with PFS and may cooperate in addition with tumor cell-intrinsic parameters (featured by the Miettinen score) to influence the clinical outcome of GIST.

Since the pioneering observation describing an inverse correlation between NK cell cytotoxicity and cancer incidence (24), many independent groups reported that intratumoral NK cell infiltrates in solid tumors are rare, anergic or irrelevant. Using tumor tissue microarrays (TMA), Sconocchia et al. found that 71% of melanomas, 97% of breast cancers, 92% of renal cell carcinoma and 92% of hepatocellular carcinomas contained no CD56+ cell infiltrate, and similar results were obtained using anti-NKp46 or anti-CD57 antibodies (25). Slinfluff’s corroborated these data showing that metastatic melanoma are devoid of NK cells and are rather infiltrated by T and B cells (26). Cremer’s group carefully studied the profile of NK TILs in early-stage non-small cell lung cancers. Few NK cells were observed in the invasive margin, mostly CD56dim that exhibited reduced levels of activating receptors (NKp30, NKp80, CD16, DNAM-1) and a decreased degranulation and cytokine secretion (27). These findings were in accordance with other observations in similar cancer types even though in some instances, NK cells were enriched in CD56bright cells (28, 29). In HPV-related tumors, such as cervix carcinoma and high-grade squamous intraepithelial lesions, loss of NCR expression on NK cells correlated with their weak cytotoxicity, HPV-16 infection and advanced clinical stage (30). Even in breast cancers that are prone to be controlled by NK cells (31) and contain NK TILs enriched in CD56bright cells, NK TILs exhibited low expression of NCR and defective functions (ADCC, cytokine release, degranulation) that inversely correlated with the Nottingham prognostic index and upregulated NKG2A inhibitory receptors (32). In contrast, in kidney cancers, high NK TILs...
content was associated with high CD16 expression on NK cells but low cytotoxic function after ex vivo reactivation using IL-2 or NKp46 stimulation (33). More importantly, the presence of intratumoral NK cells was not associated with clinical outcome at early stages of lung (27) and colon (25) cancers.

So, what might be the reason why GIST fail to tolerize NK cells? Chronic NKR ligand stimulation by tumor cells may account for the down regulation of DNAM1 or NKG2D on NK cells and perturbed NK TIL functions, as this has been shown for breast and ovarian cancers (32, 33). GIST cells failed to express most of the NKR ligands at diagnosis (Fig. 4D), which may explain that NK cells conserve high expression of NKR. Moreover, our high content multiplex cytokine/chemokine array analysis on fresh GIST (not shown) confirmed the local secretion of stem cell factor (SCF) and stem cell growth factor (SCGF) (34, 35), which both could contribute to in situ homeostasis or differentiation of NK TILs.

Does oncogene inhibition (through IM) contribute to strengthening NK cell immunosurveillance against GIST? Even though IM treatment leads to MHC class I loss in vivo, it is unlikely that the IM effects are cell-autonomous and result in enhanced target cell recognition by NK TILs, for several reasons. First, WT KIT GIST are particularly enriched in NK cells (Fig. 1E). Second, incubation of IM-sensitive versus IM-resistant KIT transduced tumor cells in the presence of IM did not reveal the capacity of IM to modulate degranulation or cytokine release (Fig. 4F). However, as for p53-dependent senescence (36), KIT inactivation by IM may modulate chemokine release, specifically during the dialogue between NK cells and KIT-expressing tumor cells. Of note, the Cxcl9 and Cxcl10 gradients were inversed by IM treatment from an initial preference for the peripheral stroma to a later preference for the tumor core (SR, unpublished data), and this may
contribute to the intratumoral homing of NK TILs. Moreover, KIT inhibition might reduce IDO concentrations, thereby reversing the intratumoral accumulation of Foxp3+ cells [(19) and Fig. 3D] and hence unleash NK cell effector functions (37).

Which immune biomarker does prevail as a prognostic biomarker in the course of GIST treated with first-line IM therapy? In contrast to soft tissue sarcoma, GIST are heavily infiltrated by leukocytes composed of all three subsets (CD4, CD8, NK) and relatively low numbers of Foxp3+ CD4+ T cells (Fig. 2A, Supplemental Fig.2 and (19)). NK TIL accumulation may, at least in part, be driven through cell-autonomous features, given its significant association with the Miettinen score (gastric localization and low mitotic index, Fig. 1F, left panel and Supplemental Fig. 1) and the WT status of KIT gene (Fig.1E, left panel). Nevertheless, NK cell infiltrates represent an independent prognostic factor that adds prognostic information to the Miettinen score for optimally resected primary GIST (Fig. 5-6). NKp30 is the only NKR to be down modulated on local and circulating NK cells, and the expression of functionally distinct NKp30 isoforms dictates PFS after IM therapy (18). In addition, T cells appear to play a major role in the immunosurveillance of GIST harboring exon 11 mutations. The Foxp3 infiltrates (as measured in IHC) were positively correlated with the high-risk Miettinen score (Fig 1F, right panel) and to a lesser extent with the mutational status (exon 11 mutations of KIT) (Fig 1E, right panel). The Foxp3 infiltrates strongly decreased post-IM (Fig. 3D). Foxp3+ cells were as frequent in primary as in metastatic lesions (Fig. 1A, right panel and 3D). These data are in accordance with Balachandran et al (19) showing that IM treatment (mainly in GIST harbouring KIT exon 11 mutations) was accompanied by Treg apoptosis secondary to the reduction of IDO. When we analyzed the prognostic value of Foxp3 infiltrates in our cohort, we found that
intratumoral Treg numbers were not associated with PFS in primary GIST. However, when stratifying the patients according to the absence or presence of adjuvant IM treatment post-surgery, we found that the accumulation of Foxp3+ cells and the loss of NKp46+ cells were both negative predictors of IM responses but were not prognostic markers (not shown). Further investigations on larger cohorts will be required to validate these data. Given that CD3+ T cell infiltrates represent an independent prognostic marker for PFS in localized GIST, it is tempting to speculate that they may shape the tumor microenvironment at early stages (Fig. 1, Supplemental Fig. 1, Fig. 5, Fig. 6 and Table 2). Given that both T and NK subsets did not colocalize within the same areas of the tumor (Fig. 1A-B and Fig. 3B-C), did not correlate in the frequency with each other (Fig. 1C) and added prognostic values to different mutations (Fig. 5-6), we postulate that they should be considered as cooperating, yet independent factors that both influence the clinical outcome of localized GIST.

What are the current recommendations for assessing the risk of relapse after resection of a primary localized GIST? They are based on five parameters: tumor size, tumor location, mitotic index (22), mutational status of GIST (38), and more recently tumor rupture during surgery (39). However, new predictors are currently being investigated, taking into account the accumulation of chromosomal aberrations (40-42), the inactivation of the tumor suppressor gene CDKN2A, the co-deletion of the gene coding for the methylthio-adenosine phosphorylase and the downregulation of the cell cycle inhibitor p27 (43-45). Prospective studies must evaluate the predictive role of T and NK cell infiltrates and to correlate them with the aforementioned intrinsic features of GIST. Based on the results obtained in this limited series of patients, we anticipate that the accurate quantification of the density and function of distinct lymphocyte subsets will refine current
methods of risk stratification in GIST and hence possibly guide therapeutic decisions, including those affecting present and future immunotherapies.

METHODS

Patients and specimens. The immunohistochemical study was carried out on a total of 91 paraffin-embedded GIST specimen (67 from the localized GIST cohort (57 No IM; 10 post IM) and 24 from the metastatic GIST cohort (10 no IM; 14 post IM)). Patients’ characteristics are depicted in Supplemental Table 1. The immunomonitoring studies on fresh tumors were prospectively performed on 14 GIST patients (9 at diagnosis and 5 post-IM) and 6 Soft Tissue Sarcoma (STS) patients enrolled for surgery (Supplemental Table 2). Patients’ samples were provided by the Gustave Roussy Institute (Villejuif, France), the University Hospital Virgen de las Nieves (Granada, Spain), the Léon Bérard Centre (Lyon, France), the Bergonié Institute (Bordeaux, France), the University Hospital Jean Minjoz (Besançon, France) and the University Hospital Dupuytren (Limoges, France). An informed written consent was obtained from patients according to the local ethical committee. Heparinized blood was drawn from patients before surgery for the immunomonitoring study. Clinical responses were assessed by computed tomography (CT) scan and the responses were classified according to the RECIST criteria.

Study Approval. Study with GIST and STS specimen were approved by the local Ethic Committee (2007-A00923-50) with written informed consent received from patients.
**GIST tumor dissociation and flow cytometry.** Fresh tumors were dissociated within 12h post-surgery following the gentle MACS Dissociator protocol (Miltenyi). Briefly, resected tumors were cut into small pieces, placed in tube C with the dissociation buffer (RPMI 1640, 100U Pen/Strep, 50U/mL Collagenase IV, 30U/mL DNase, 280U/mL hyaluronidase) and processed with the h_tumor_01 program on the gentle MACS dissociator. The resulting cell suspension was filtered through a 70 μm cell strainer (BD Biosciences), washed in PBS and cell viability was estimated by Hayem Blue. Tumor cell suspensions were analyzed by 12-color flow cytometry and NK cells were defined as CD45+CD3-CD56+ and tumor cells as CD45-CD34+KIT+. The NK receptor and tumor ligand antibodies included in this study are listed in Supplementary Table 3. Cells were incubated with conjugated antibodies for 20 minutes at 4°C, washed and fixed with 1% paraformaldehyde.

For intracellular cytokine staining, patients’ PBMC and TILs were seeded in 96-round bottom plates at 1x10^5 cells/well in RPMI + 10% AB serum with PMA/Iono (10ng/mL and 250ng/mL respectively, Calbiochem) plus Golgi Stop (4uL/6mL, BD Bioscience) for 2 hours at 37°C. Cells were fixed and permeabilized with Fix/Perm reagents (eBioscience) following manufacturer’s protocol. Stained cells were acquired within 24 hours on Cyan Flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

**Immunohistochemistry (IHC) staining.** Paraffin–embedded GIST specimen were assessed for the infiltration by NK, CD3, CD8, Foxp3+ cells and HLA class I positivity (EMR8-5) with the antibodies listed in Supplementary Table 3. Briefly, 3μm–thick sections of formalin-fixed, paraffin–embedded GIST specimen were mounted on poly–L–lysine-coated slides, deparaffinized for antigen retrieval and then rehydrated through graded alcohols to water.
Retrieval buffers consisted in Tris–EDTA buffer (10mM Tris, 1mM EDTA) pH9 for NKp46, pH8 for CD8 staining; 0.01M citrate buffer pH10 for Foxp3 and pH7 for EMR8-5. Slides were incubated for 30 minutes in a 98 °C water bath. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxidase (DAKO) for 10 minutes and non-specific protein were blocked for 15 minutes (Protein block, DAKO). The primary antibody was incubated for 1 hour, followed by the secondary Ab, polymer-peroxidase for mouse (NKp46, EMR8-5), or rabbit (CD3, CD8) monoclonal Ab (EnVision™System, DAKO) or Novolink (NOVOCASTRA) for Foxp3, incubated for 45 minutes. Peroxidases were detected with 3-amino-9-ethylcarbazole substrate (AEC, Vector Laboratories) for NKp46 and CD8 or with Di Amino Benzidine-peroxidase substrate kit (DAKO) and the sections were counterstained with Harris’s hematoxylin and dehydrated except for AEC substrate (NKp46 and CD8) where the slides were mounted using Glycergel (DAKO). Negative controls were made by substituting primary Ab with isotype controls. CD3 staining was performed on fully automated Ventana Benchmark XT system (Roche Ventana) following manufacturers’ recommendations. Positive cells were counted in 10 random x200 fields in a double-blinded fashion. For CD3 staining, slides were digitized with a slide scanner (Nikon Supercoolscan 8000 ED) and processed with Pix Cyt software enabling whole slide quantification (46).

NK/tumor cross-talk in vitro. TF-1 erythroleukemic cell line engineered to express human KIT gene either resistant (hKIT D816V or TF816, exon 17) or sensible (hKIT Δ417/8+D419Y or TF417, exon 11) to IM (kindly provided by Dr Dubreuil, Marseille, France) (23) were cultivated in RPMI supplemented with 10% fetal calf serum (FCS, PAA), 1%
Pen/Strep and 2mM Glutamine. 5x10⁵ tumor cell lines (TF417, TF816 and GIST 888) were seeded in 24 well plate overnight. The following day, IM (10⁻⁶M) was added or not and cells were incubated for 24 hours. Tumor cell viability was assessed by Annexin V/PI staining and the expression of NK cell ligands was monitored with the antibodies listed in Supplemental Table 3. In some experiments, IM-treated tumor cell lines (TF417 and TF816) were incubated with healthy volunteers’ enriched NK cells (Easy Sep kit, Stem cell Technologies) at a ratio 1:5 to monitor CD107a membrane expression by flow cytometry, following 5 hours’ incubation with 2 μl of anti-CD107a-PE and 10 μM Golgi Stop (BD Biosciences) as well as IFNγ production by commercial ELISA (BD Biosciences) following 24 hours incubation.

Statistical analyses. The Fisher’s exact test, the Chi-square test, the linear regression test and the parametric Wilcoxon test and the non-parametric Mann-Whitney test were used for comparison of the different groups. These statistical analyses were performed with the GraphPad Prism software version 5. The survival curves were plotted according to the Kaplan-Meier method, and compared using the log-rank (Mantel Cox). Multivariate analyses were performed with Spss 17.0 (IBM SPSS, Paris, France), using the Cox model.

Reference


Table 1: Prognostic factors in the series with localized GIST

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Univariate analysis</th>
<th>Cox model</th>
<th>Univariate analysis</th>
<th>Cox model</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Median PFS (months)</td>
<td>p</td>
<td>HR</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>NR</td>
<td>NR</td>
<td>163</td>
<td>NR</td>
</tr>
<tr>
<td>10-50%</td>
<td>7</td>
<td>145</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50%</td>
<td>27</td>
<td>77</td>
<td>NR</td>
<td></td>
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<tr>
<td><strong>NKp46</strong></td>
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<tr>
<td>&lt;13/hpf</td>
<td>27</td>
<td>77</td>
<td>163</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;13/hpf</td>
<td>26</td>
<td>NR</td>
<td>NR</td>
<td></td>
<td></td>
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<tr>
<td><strong>CD3</strong></td>
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<td>0.001</td>
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<td>-</td>
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<tr>
<td>&lt;=9.9%</td>
<td>26</td>
<td>45</td>
<td>NR</td>
<td>163</td>
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</tr>
<tr>
<td>&gt;9.9%</td>
<td>27</td>
<td>145</td>
<td>NR</td>
<td></td>
<td></td>
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<tr>
<td><strong>FoxP3</strong></td>
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<td>-</td>
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<tr>
<td>&gt;1.3/hpf</td>
<td>26</td>
<td>77</td>
<td>NR</td>
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</table>

NR: not reached; PFS: progression free survival; OS: overall survival; HR: hazard ratio. P values calculated with the Logrank test for univariate analysis. The following parameters were tested in univariate analysis and found not significantly correlated to survival: gender, nature of the mutation. For the Cox models, all four parameters above (Miettinen score, CD3, NKp46, FOXP3) were included in the model.
Figures legend

**Figure 1. T and NK cell infiltration in localized and metastatic GIST at diagnosis.**

*A-B. Enumeration of GIST TILs in immunohistochemistry.* (A) Representative microphotographs of immunohistochemical stainings of primary and metastatic paraffin-embedded GIST using antibodies specific for NKp46 (left), CD3 (middle) or Foxp3 (right). The number of specimen is indicated. The mean numbers of NKp46+ and Foxp3+ cells enumerated in 10 independent fields observed at x200 power of magnification are depicted per field. As for the CD3 staining, the percentages of positive cells scanned on the whole slide are indicated. (B) The precise enumeration of NKp46+ cells was performed strictly in fibrous trabeculae and in the tumor foci of primary paraffin-embedded GIST by analyzing at least 10 representative fields. The median numbers/field are represented, each dot featuring one GIST. (C-D) Correlations between CD3+ T cells and NKp46+ infiltrate (C) and between Foxp3+ cells and NKp46+ infiltrates (D). *E-F. Correlations between immune parameters and the KIT mutational status, as well as the Miettinen score.* The enumeration of NKp46, CD3 and Foxp3 cells (left, middle and right panel respectively) was correlated with the presence of KIT exon 11 mutation (WT meaning no detectable mutations in KIT gene) (E) or with the Miettinen score (low and intermediate risk versus high risk) (F). Linear regression, Wilcoxon matched paired or Mann Whitney tests: *p<0.05, **p<0.01, ***p<0.001.

**Figure 2. T and NK cell infiltrates in GIST are activated.**

*A. Flow cytometry analyses of NK and T cells in CD45+ live leukocytes from freshly dissociated GIST compared with circulating blood lymphocytes or soft tissue sarcomas (STS, Supplemental Table 2).* The percentages of NK cells or CD3+CD8+ or CD3+CD4+ or regulatory T cells contained among CD45+ live TILs in 9 primary GIST at diagnosis and 6 STS are depicted in blood and tumor beds in a paired manner. (B) The expression levels of CD69 in blood and tumor NK cells are indicated in a typical dot plot analysis (left panel) and for all 9 primary
GIST at diagnosis (right panel). (C-E) TILs were subjected to a 2 hours PMA/ionomycine stimulation after ex vivo dissociation and analyzed by flow cytometry using intracellular stainings for Th1 cytokines (IFNγ and TNFα) gating on NK (C), CD8+ (D) or CD4+ T cell subsets (E). A typical dot plot analysis is shown indicating the means±SEM of the percentages of IFNγ (upper quadrant) and TNFα (lower quadrant) positive cells obtained in all specimen. (F) Representative dot plots of CD16, CX3CR1 and NKp80 expression on CD3-CD56+ NK cells comparing paired blood and GIST tumor NK cell phenotypes (overlay of tumor (blue) and blood (red) NK cells). (G) All NKR and KIR were analyzed in flow cytometry gating on infiltrating tumor CD3-CD56+CD45+ NK cells in 14 GIST (9 at diagnosis and 5 post IM) and 6 STS. Wilcoxon matched paired or Mann Whitney test: *p<0.05, **p<0.01, *** p<0.001.

**Figure 3. Effects of IM on the dynamics of T and NK cell subsets in GIST.**
The effects of IM on GIST TILs infiltrates were evaluated by immunohistochemistry (as shown in Fig. 1). (A) A representative micrograph picture of NKp46 staining (at x200 power of magnification) is shown in fibrous trabeculae (FT) and in tumor nests (TN). (B) The precise enumeration of NKp46+ cells was performed strictly in fibrous trabeculae and in the tumor nests by analyzing at least 10 representative fields. The median numbers/field are represented, each dot featuring one GIST before and after IM in primary localized or metastatic GIST. The number of specimen is indicated on the graph. (C-D) Similar analyzes were performed for CD3+(C) and Foxp3+(D) infiltrates from the same specimen. Mann-Whitney test: *p<0.05, **p<0.01, *** p<0.001.

**Figure 4. Immunoselection against MHC class I positive tumor cells associated with IM therapy.**
A-B. Immunohistochemical analyses of primary paraffin-embedded GIST using the EMR85 antibody. The percentages of GIST presenting with a negative (-), positive (+) or heterogeneous (+/-) staining at diagnosis (n=32) and after neoadjuvant IM (n=10) are indicated (A). The enumeration of CD8+ and NKp46+ cells in all the EMR85 positive versus negative tumors is represented (B, left and right panels). (C) A representative micrograph picture of a heterogeneous (+/-) staining using EMR85 Ab and the adjacent tissue section
stained with H&E, anti-CD3 and anti-CD8 Ab. Enumeration of CD3+ cells in EMR85 positive versus negative areas in all heterogenous cases is represented in percentages (C, right panel). D. Flow cytometry analyses of MHC class I, MHC class II, and MHC like molecules as well as B7 family members on freshly dissociated primary GIST. Ratio of MFI for 9 specimen analyzed at diagnosis and 5 specimen after neoadjuvant IM are depicted. E-F. Direct effects of IM on tumor cells in vitro. (E) MHC class I, B7-H6, MICA/B and isotype control in TF417 (sensitive to IM) versus TF816 (resistant to IM) exposed or not to IM in vitro. (F) Effector functions of allogeneic NK cells (enriched from HV) against TF417 versus TF816 after a coculture in the presence or absence of IM. Membrane expression of CD107a and IFNγ secretion were analyzed by flow cytometry and ELISA respectively. Unpaired-T Test or Mann Whitney test: *p<0.05, **p<0.01.

Figure 5. Prognostic value of T and NK cell infiltrates in localized GIST.
(A-C) Progression-free survival (PFS) of 53 localized GIST patients according to the median values of NKp46, CD3 or Foxp3 positive cells infiltrating the tumor at diagnosis (left, middle and right panels). (D) Kaplan Meier curves of PFS obtained by stratifying the entire cohort of primary GIST into four groups according to the median of NKp46+ and CD3+ cells. Multivariate analyses are presented in Table 1.

Figure 6. Impact of immune parameters on localized GIST with high-risk Miettinen scores.
Kaplan Meier curves of PFS obtained by stratifying the whole cohort of primary GIST at diagnosis into several groups according to the median of NKp46 or CD3 positive cells and the Kit mutation (A-B, N=46, with N=12 KIT WT and N=34 KIT exon 11 GIST bearing patients) or the Miettinen score alone (C, N=53 with N=19 low, N=8 intermediate and N=26 high risk) or a combination of Miettinen scores and immune parameters (D-E). Log-Rank (Mantel Cox): *p<0.05, **p<0.01, *** p<0.001.
**Figure 1**
Figure 2
Figure 3
**Figure 4**

A.

B.

C.

D.

E.

F.
Figure 5
Figure 6
Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors

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