Abstract

Cancer immunosurveillance relies on effector/memory tumor-infiltrating CD8+ T cells with a T-helper cell 1 (Th1) profile. Evidence for a natural killer (NK) cell-based control of human malignancies is still largely missing. The KIT tyrosine kinase inhibitor imatinib mesylate 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 (TIL) 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 despite imatinib mesylate 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 imatinib mesylate 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 patients with GIST.

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).

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Indeed, 70% to 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 (WT) KIT, 5% to 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; ref. 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 was first administered to a GIST-bearing patient in 2001 (5). Imatinib mesylate 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 imatinib mesylate is 5 years and 34% of the patients survive more than 9 years (11, 12). In addition, adjuvant imatinib mesylate decreases the risk of relapse after resection of a localized GIST (6). However, even long-term imatinib mesylate treatment fails to eradicate GIST cells in both advanced and localized adjuvant settings, perhaps because imatinib mesylate has mostly cytostatic effects or because GIST stem cells do not require KIT signaling for their survival (13). Moreover, continuous imatinib mesylate exposure can lead to the selection imatinib mesylate–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 succinate dehydrogenase-deficient tumors).

Another potential strategy consists if mobilizing the immune system against GIST during/after imatinib mesylate therapy. Indeed, immunostimulatory bystander or off-target effects of distinct cytotoxic compounds reportedly contribute to their anticancer effects (15). We found that imatinib mesylate can promote a KIT-dependent cross-talk between dendritic and natural killer (NK) cells, leading to IFN-γ production by NK lymphocytes, both in mice and in humans (16). Importantly, in advanced GIST, NK cell activation following 2 months of imatinib mesylate 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 patients with metastatic GIST treated with imatinib mesylate (18). Beyond NK cells, T cells may influence the course of GIST. Balachandran and colleagues reported the discovery that, in mouse GIST models, the antitumor effects of imatinib mesylate relied on the contribution of CD8+ T lymphocytes (19). Thus, inhibition of oncopgenic KIT by imatinib mesylate 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 2 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.

**Materials and 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 imatinib mesylate; 10 post-imatinib mesylate) and 24 from the metastatic GIST cohort (10 no imatinib mesylate; 14 post-imatinib mesylate)]. Patients’ characteristics are depicted in Supplementary Table S1. The immunomonitoring studies on fresh tumors were prospectively conducted on 14 patients with GIST (9 at diagnosis and 5 post-imatinib mesylate) and 6 patients with soft tissue sarcoma (STS) enrolled for surgery (Supplementary Table S2). 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 (Limosges, 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 scan and the responses were classified according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria.

**Study approval**

Study with GIST and STS specimen was 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 12 hours postsurgery 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, 100 U penicillin/streptomycin, 50 U/mL Collagenase IV, 30 U/mL DNase, and 280 U/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+ CD3+ KIT+. The NK receptor and tumor ligand antibodies included in this study are listed in Supplementary Table S3. 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 tumor-infiltrating lymphocytes (TIL) were seeded in 96-round bottom plates at 1 × 10^5 cells per well in RPMI + 10% AB serum with phorbol 12-myristate 13-acetate (PMA)/ionomycin (10 ng/mL and 250 ng/mL, respectively; Calbiochem) plus Golgi Stop (4 μL/mL; 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).

**Immunohistochemical 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 S3. Briefly, 3-μm 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 of Tris–EDTA buffer (10 mmol/L Tris and 1 mmol/L EDTA) pH 9 for NKp46, pH 8 for CD8 staining; 0.01 mol/L citrate buffer pH 10 for Foxp3 and pH 7 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 nonspecific protein were blocked for 15 minutes (Protein Block; Dako). The primary antibody was incubated for 1 hour, followed by the secondary antibody, polymer-peroxidase for mouse (NKp46, EMR8-5), or rabbit (CD3, CD8) monoclonal antibody (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 diaminobenzidine-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 antibody with isotype controls. CD3 staining was carried out on fully automated Ventana Benchmark XT system (Roche Ventana) following manufacturers’ recommendations. Positive cells were counted in 10 random ×200 fields in a double-blind 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 (22).

**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 (hKITΔ17/8+D419T or TF417, exon 11) to imatinib mesylate (kindly provided by Dr. P. Dubreuil, Institut Paoli-Calmettes, Marseille, France; ref. 23) were cultivated in RPMI supplemented with 10% fetal calf serum (PAA), 1% penicillin/streptomycin and 2 mmol/L glutamine. A total of 5 × 10^5 tumor cell lines (TF417, TF816, and GIST 888) were seeded in 24-well plate overnight. The following day, imatinib mesylate (10^-6 mol/L) was either added or not added and cells were incubated for 24 hours. Tumor cell viability was assessed by Annexin V/propidium iodide (PI) staining and the expression of NK cell ligands was monitored with the antibodies listed in Supplementary Table S3. In some experiments, imatinib mesylate–treated tumor cell lines (TF417 and TF816) were incubated with healthy volunteers’ enriched NK cells (Easy Sep kit; STEMCELL Technologies) at a ratio 1:5 to monitor CD107a membrane expression by flow cytometry, following 5 hours of incubation with 2 μL of anti-CD107a-PE and 10 μmol/L Golgi Stop (BD Biosciences) as well as IFN-γ production by commercial ELISA (BD Biosciences) following 24 hours incubation.

**Statistical analyses**

The Fisher exact test, the χ² test, the linear regression test, and the parametric Wilcoxon test and the nonparametric Mann–Whitney test were used for comparison of the different groups. These statistical analyses were conducted 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 conducted with SPSS 17.0 (IBM SPSS), using the Cox model.

**Results**

**NK cell infiltrates in GIST**

We conducted a retrospective immunohistochemical analysis of a cohort of 57 localized and 10 metastatic GIST at diagnosis (imatinib mesylate–free; Supplementary Table S1) using antibodies directed against CD3, Foxp3, and NKp46 aimed at identifying T lymphocytes, regulatory T cells (Treg), and NK cells, respectively. The NKp46–specific staining resided in fibrous trabeculae (Fig. 1A, left and Fig. 1B), whereas CD3– and Foxp3– cells were mostly localized in tumor foci (Fig. 1A, middle and right). The primary tumor of metastatic GIST exhibited similar patterns of TILs (Fig. 1A). There was no significant correlation between the numbers of T, Tregs, and NK cells (Fig. 1C and D). In the absence of imatinib mesylate therapy, harboring a KIT exon 11 mutation or a high Miettinen score is associated with dismal prognosis (24). In localized GIST, NK TILs were 3 times less frequent in GIST bearing a KIT exon 11 mutation than in WT GIST (Fig. 1E, left; median of 6 vs. 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, P = 0.04; and right, 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 (Supplementary Fig. S1A and S1B, left; P = 0.04 and 0.01, respectively), whereas the frequency of CD3^+^ cells inversely correlated with tumor size (Supplementary Fig. S1C, middle and right).

To investigate the functional phenotype of NK TILs, we conducted a comprehensive flow cytometry analysis of NK cells purified from 14 freshly harvested and dissociated localized GIST tumors (9 at diagnosis and 5 post-imatinib mesylate), comparing them with autologous circulating NK cells or to NK cells purified from 6 STS (Supplementary Table S2). GIST
Figure 1. T and NK cell infiltration in localized and metastatic GIST at diagnosis. A and B, enumeration of GIST TILs in IHC. 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 ×200 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 conducted 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 and D, correlations between CD3⁺ T cells and NKp46⁺ in infiltrate (C) and between Foxp3⁺ cells and NKp46⁺ in infiltrates (D). E and 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, 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 vs. high risk; F). Linear regression, Wilcoxon matched paired, or Mann–Whitney tests: *, P < 0.05; **, P < 0.01;***, P < 0.001. NS, nonsignificant.
contained denser leukocytic infiltrates than STS (20% ± 6% of live cells vs. 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 as most TILs were CD4+ T cells (Fig. 2A and Supplementary Fig. S2). The proportion of CD3+CD4+CD25high/CD127− Tregs was not enhanced in TILs compared with blood (Fig. 2A and Supplementary Fig. S2). GIST NK TILs expressed the activation marker CD69 ex vivo (Fig. 2B) and produced IFN-γ (and to a lesser extent TNF-α) after a brief (2 hours) restimulation with PMA/ionomycine (Fig. 2C). CD3+ TILs also produced T-helper cell 1 (Th1) cytokines in similar conditions (Fig. 2D and E). The phenotype of NK TILs in GIST was homogeneous

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 STS (Supplementary Table S2). The percentages of NK cells or CD3+CD8− or CD3+CD4+ or Tregs 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) and for all 9 primary GIST at diagnosis (right). 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 (IFN-γ and TNF-α) gating on NK (C), CD8+ (D), or CD4+ T-cell subsets (E). A typical dot plot analysis is shown, indicating the mean ± SEM of the percentages of IFN-γ (top quadrant) and TNF-α (bottom 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 killer-cell immunoglobulin-like receptor were analyzed in flow cytometry gating on infiltrating tumor CD3+CD56+CD45− NK cells in 14 GIST (9 at diagnosis and 5 post-imatinib mesylate) and 6 STS. Wilcoxon matched paired or Mann–Whitney test: **, P < 0.01; *** P < 0.001. NS, nonsignificant.
with little interindividual or intraintividual (pre- vs. post-imatinib mesylate) variations as most GIST NK TIL were CD3−CD56brightCD94−NKp80−CD16−NK2G2A−CD158− (Fig. 2F and G; but expressed variable levels of NKp90/CXCR3/CX3CR1) cells (Fig. 2G). In STS, NK TILs were not only rare but also CD3−CD56dimCD16+ 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 CD56bright NK cells that are devoid of inhibitory receptors and are localized at the tumor borders. Moreover, GIST TILs contained activated T(H)1 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 imatinib mesylate therapy on GIST TIL. Patients received 400 mg imatinib mesylate daily for an average time of 10.7 months. Although the density of NKp46 infiltrates did not change in fibrous trabeculae (Fig. 3A and B), the frequency of NKp46+ cells infiltrating the core of localized or metastatic GIST increased after imatinib mesylate treatment (Fig. 3B, right; \( P = 0.004 \) for localized and \( P = 0.001 \) for metastatic GIST). In contrast, Foxp3+ cells significantly decreased, whereas CD3+ cell numbers did not change (Fig. 3C and D). Hence, the ratio NKp46/Foxp3 in the tumor nests markedly increased by 4-fold after imatinib mesylate.

Because both T and NK cell education and activation are dictated by MHC class I molecules, we conducted immunohistochemical analyses of a total of 42 localized GIST (surgery alone or followed by adjuvant imatinib mesylate, \( n = 32 \); neoadjuvant imatinib mesylate followed by surgery, \( n = 10 \)) using the EMB8.5 antibody specific for a nonpolymorphic epitope of the heavy chain of MHC class I molecules. Although most GIST at diagnosis homogeneously express MHC class I molecules, up to 30% GIST fully lost MHC class I expression upon imatinib mesylate 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; \( P = 0.03 \)) and areas (Fig. 4C), but disappeared from MHC class I−negative tumors or areas (Fig. 4B, left and Fig. 4C). In contrast, NK cells were not enriched in MHC class I−negative tumors or areas (Fig. 4B, right). 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 imatinib mesylate. The level of expression of MHC class I molecules was higher in GIST at diagnosis compared with GIST treated with neoadjuvant imatinib mesylate [mean ratio mean fluorescence intensity (MFI) staining/MFI isotype control: 11 ± 4 vs. 2 ± 1, respectively; Fig. 4D; \( P = 0.042 \)], whereas 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 patients with neoadjuvant GIST enrolled in this study, we found MHC class I loss variants in all (4 of 4) patients that were disease-free at 7 years postdiagnosis, whereas only half of the patients (3 of 6) relapsing or dying of their disease manifested loss of MHC class I expression.

![Figure 3. Effects of imatinib mesylate (IM) on the dynamics of T and NK cell subsets in GIST. The effects of imatinib mesylate on GIST TILs infiltrates were evaluated by IHC (as shown in Fig. 1). A, a representative micrograph picture of NKp46 staining (magnification, >200) is shown in fibrous trabeculae (FT) and in tumor nests (TN). B, the precise enumeration of NKp46+ cells was conducted 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 imatinib mesylate in primary localized or metastatic GIST. The number of specimen is indicated on the graph. C and D, similar analyses were conducted for CD3+ (C) and Foxp3+ (D) infiltrates from the same specimen. Mann–Whitney test: *, \( P < 0.05 \); **, \( P < 0.01 \), NS, nonsignificant.](image-url)
The reduction of MHC class I expression by GIST tumors did not result from direct oncogene inhibition by imatinib mesylate. We took advantage of imatinib mesylate–sensitive (TF417 cells bearing an exon 11 Kit mutation) and imatinib mesylate–resistant (TF816 cells with an exon 17 Kit mutation) KIT-transduced erythroleukemic cells that we incubated in the presence of imatinib mesylate and allogeneic NK cells (from normal volunteers). Imatinib mesylate 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 imatinib mesylate and the NK cell effector functions could be observed (Fig. 4F).

We conclude that long-term treatment with imatinib mesylate 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.
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 imatinib mesylate in adjuvant setting. NK and CD3 cell infiltrates were both associated with a reduced relapse rate (Fig. 5A and B; \( P = 0.001 \) and 0.007, respectively), whereas 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 (HR = 2.26; \( P = 0.05 \)) and the NKp46 scores (HR = 0.2; \( P = 0.01 \)), were independent prognostic factors for progression-free survival (PFS; Table 1). Neither gender nor

### Table 1. Prognostic factors in the series with localized GIST

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**NOTE:** \( P \) values calculated with the log-rank 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 4 parameters above (Miettinen score, CD3, NKp46, and FOXP3) were included in the model. Bold italic values are the statistically significant \( P \) and HR values of the univariate analysis and Cox model. Abbreviations: NR, not reached; OS, overall survival.
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\textsuperscript{low}CD3\textsuperscript{low} and had a dismal prognosis (Fig. 5D; $P = 0.0005$).

Stratification of patients with GIST based on the oncogenic KIT mutation and the NKp46 score identified a subset of 10% of patients that were KIT\textsuperscript{WT} NKp46\textsuperscript{low} (33% of all KIT WT GIST) with a particularly high relapse rate regardless of imatinib mesylate adjuvant therapy (Fig. 6A; $P = 0.006$). Moreover, stratification of patients with GIST based on the oncogenic KIT mutation and the CD3 score identified a subset of 33% of patients that were KIT\textsuperscript{exon11} CD3\textsuperscript{low} (44% of all exon 11–mutated GIST) with a particularly high relapse rate regardless of imatinib mesylate adjuvant therapy (Fig. 6B; $P = 0.03$). As expected (24), the Miettinen score alone has an impact on survival with a 5-year median of PFS for patients bearing a high score, whereas PFS was unreach for patients with GIST with Miettinen\textsuperscript{low/intermediate} score (Fig. 6C; $P = 0.0062$). Combining both Miettinen and NKp46 parameters, we identified a subgroup of patients (about one half of patients with Miettinen\textsuperscript{high} GIST) presenting with a Miettinen\textsuperscript{high} NKp46\textsuperscript{high} score and a reduced relapse rate, similar to patients with Miettinen\textsuperscript{low/intermediate} scores (Fig. 6D; $P < 0.0001$), regardless of imatinib mesylate adjuvant therapy (given that 7 of 11 patients underwent surgery only). In contrast, patients with GIST with a Miettinen\textsuperscript{high} and NKp46\textsuperscript{low} score exhibited a short PFS (0.91 year vs. 5 years for high Miettinen score alone; Fig. 6D), independently of adjuvant-imatinib mesylate therapy (given that 53% of Miettinen\textsuperscript{high} NKp46\textsuperscript{low} underwent surgery followed by adjuvant imatinib mesylate therapy). The CD3 score also affected the fate of high Miettinen\textsuperscript{high} GIST in thus far that a subset of 22% GIST (harboring a Miettinen\textsuperscript{high} CD3\textsuperscript{low} score) had a dismal prognosis (Fig. 6E; $P < 0.0001$). Thus, in localized GIST, both the CD3\textsuperscript{+} and NKp46\textsuperscript{+} 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 seems 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 (25), many independent groups reported that intratumoral NK cell infiltrates in solid tumors are rare, anergic, or irrelevant. Using tumor tissue microarrays, Scnoca and colleagues 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 (26). Erdag and colleagues corroborated these data showing that metastatic melanoma are devoid of NK cells and are rather infiltrated by T and B cells (27). Platonova and colleagues 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, and DNAM-1) and a decreased degranulation and cytokine secretion (28). These findings were in accordance with other observations in similar cancer types even though in some instances, NK cells were enriched in CD56bright cells (29, 30). In human papillomavirus (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 (31). Even in breast cancers that are prone to be controlled by NK cells (32) and contain NK TILs enriched in CD56dim that exhibited reduced levels of activating receptors (NKp30, NKp80, CD16, and DNAM-1) and a decreased degranulation and cytokine secretion (28). These findings were in accordance with other observations in similar cancer types even though in some instances, NK cells were enriched in CD56bright cells (29, 30). In human papillomavirus (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 (31).

In breast cancers that are prone to be controlled by NK cells (32) and contain NK TILs enriched in CD56bright cells, NK TILs exhibited low expression of NCR and defective functions [antibody-dependent cell-mediated cytotoxicity (ADCC), cytokine release, and degranulation] that inversely correlated with the Nottingham prognostic index and upregulated NKG2A inhibitory receptors (33). In contrast, in kidney cancers, high NK TIL content was associated with high CD16 expression on NK cells but low cytotoxic function after ex vivo reactivation using interleukin (IL)-2 or NKp46 stimulation (34). More importantly, the presence of intratumoral NK cells was not associated with clinical outcome at early stages of lung (28) and colon (26) cancers.

So, what might be the reason why GIST fail to tolerize NK cells? Chronic NK cells receptor (NKR) ligand stimulation by tumor cells may account for the downregulation of DNAM1 or NKG2D on NK cells and perturbed NK TIL functions, as this has been shown for breast and ovarian cancers (33, 34). 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 and stem cell growth factor (35, 36), which both could contribute to in situ homeostasis or differentiation of NK TILs.

Does oncogene inhibition (through imatinib mesylate) contribute to strengthening NK cell immunosurveillance against GIST? Even though imatinib mesylate treatment leads to MHC class I loss in vivo, it is unlikely that the imatinib mesylate 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 imatinib mesylate–sensitive versus imatinib mesylate–resistant KIT transduced tumor cells in the presence of imatinib mesylate did not reveal the capacity of imatinib mesylate to modulate degranulation or cytokine release (Fig. 4F). However, for p53-dependent senescence (37), KIT inactivation by imatinib mesylate may modulate chemokine release, specifically during the dialogue between NK cells and KIT-expressing tumor cells. Of note, the Cxcl9 and Cxcl10 gradients were inversely by imatinib mesylate treatment from an initial preference for the peripheral stroma to a later preference for the tumor core (S. Rusakiewicz; 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 (Fig. 3D; ref. 19), and hence unleash NK cell effector functions (38).

Which immune biomarker does prevail as a prognostic biomarker in the course of GIST treated with first-line imatinib mesylate therapy? In contrast to STS, GIST are heavily infiltrated by leukocytes composed of all 3 subsets (CD4, CD8, and NK) and relatively low numbers of Foxp3+ CD4+ T cells (Fig. 2A; Supplementary Fig. S2; ref. 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; Supplementary Fig. S1) and the WT status of KIT gene (Fig. 1E, left). Nevertheless, NK cell infiltrates represent an independent prognostic factor that adds prognostic information to the Miettinen score for optimally resected primary GIST (Figs. 5 and 6). NKP30 is the only NKR to be downmodulated on local and circulating NK cells, and the expression of functionally distinct NKP30 isoforms dictates PFS after imatinib mesylate therapy (18). In addition, T cells seem 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) and to a lesser extent with the mutational status (exon 11 mutations of KIT; Fig. 1E, right). The Foxp3 infiltrates strongly decreased post-imatinib mesylate (Fig. 3D). Foxp3+ cells were as frequent in primary as in metastatic lesions (Fig. 1A, right and 3D). These data are in accordance with Balachandran and colleagues (19) showing that imatinib mesylate treatment (mainly in GIST harboring 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 thatintratumoral Treg numbers were not associated with PFS in primary GIST. However, when stratifying the patients according to the absence or presence of adjuvant imatinib mesylate treatment, we found that the accumulation of Foxp3+ cells and the loss of NKp46+ cells were both negative predictors of imatinib mesylate responses but were not prognostic markers (not shown). Further investigations on larger cohorts will be required to validate these data. Given that CD8+ 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; Supplementary Figs. S1, S5, and S6; Supplementary Table 2). Given that both T and NK subsets did not colocalize within the same areas of the tumor (Figs. 1A and B and 3B and C), did not correlate in the frequency with each other (Fig. 1C) and added prognostic values to different mutations (Figs. 5 and 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 5 parameters: tumor size, tumor location, mitotic index (24), mutational status of GIST (39), and more recently tumor rupture during surgery (40). However, new predictors are currently being investigated, taking into account the accumulation of chromosomal aberrations (41–43), the inactivation of the tumor suppressor gene CDKN2A (44–46). Prospective studies must evaluate the predictive role of T-cell infiltrates and to correlate them with the aforementioned intrinsic features of GIST. On the basis of 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.

Disclosure of Potential Conflicts of Interest

P. Dubreuil is a consultant/advisory board member of AB Science. J.-F. Emile is consultant/advisory board member of Novartis and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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