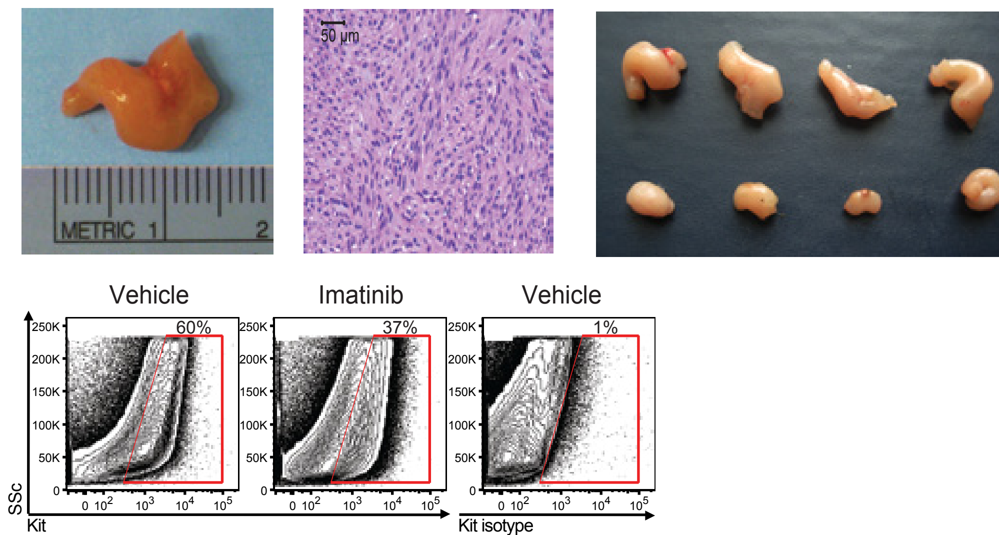
SUPPLEMENTARY FIGURES

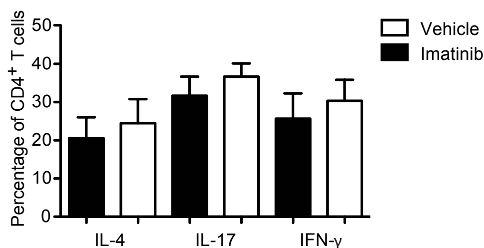
Imatinib potentiates anti-tumor T cell responses in gastrointestinal stromal tumor through the inhibition of Ido
Vinod P. Balachandran, Michael J. Cavnar, Shan Zeng, Zubin M. Bamboat, Lee M. Ocuin, Hebroon Obaid, Eric C. Sorenson, Rachel Popow, Charlotte Ariyan, Ferdinand Rossi, Peter Besmer, Tianhua Guo, Cristina R. Antonescu, Takahiro Taguchi, Jianda Yuan, Jedd D. Wolchok, James P. Allison, Ronald P. DeMatteo

1

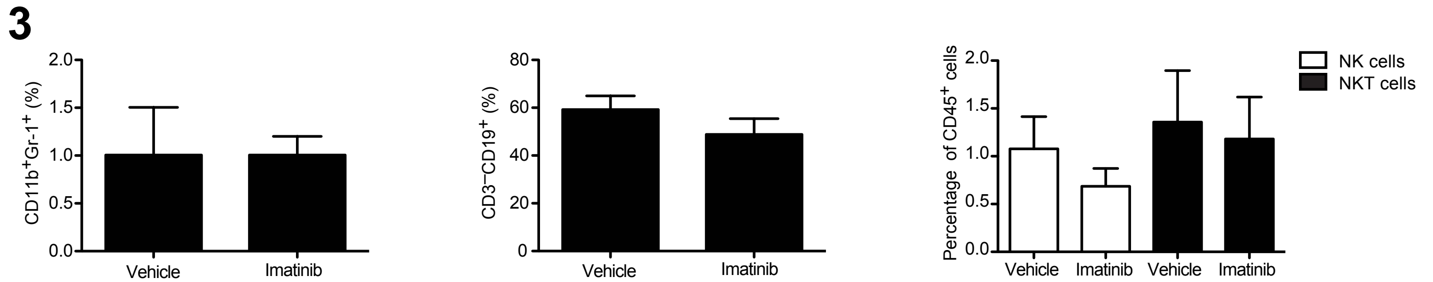


Mouse GIST develops as a discrete nodular mass in the cecum that can be easily resected and weighed (top left panel). Hematoxylin and eosin staining of mouse GIST demonstrates a spindle-shaped morphology of tumor cells that resembles human GIST (top middle panel). Tumors from GIST mice treated with vehicle (top right panel, top row) or imatinib (top right panel, bottom row) for 7 days. Only tumor tissue is shown as the tumors were separated from the intestine. Flow cytometric analysis of tumors after 7 days of treatment with vehicle or imatinib (bottom panel). Numbers indicate the percentage of tumor cells ($CD45^{-}Kit^{+}$) as a frequency of live $CD45^{-}$ cells.

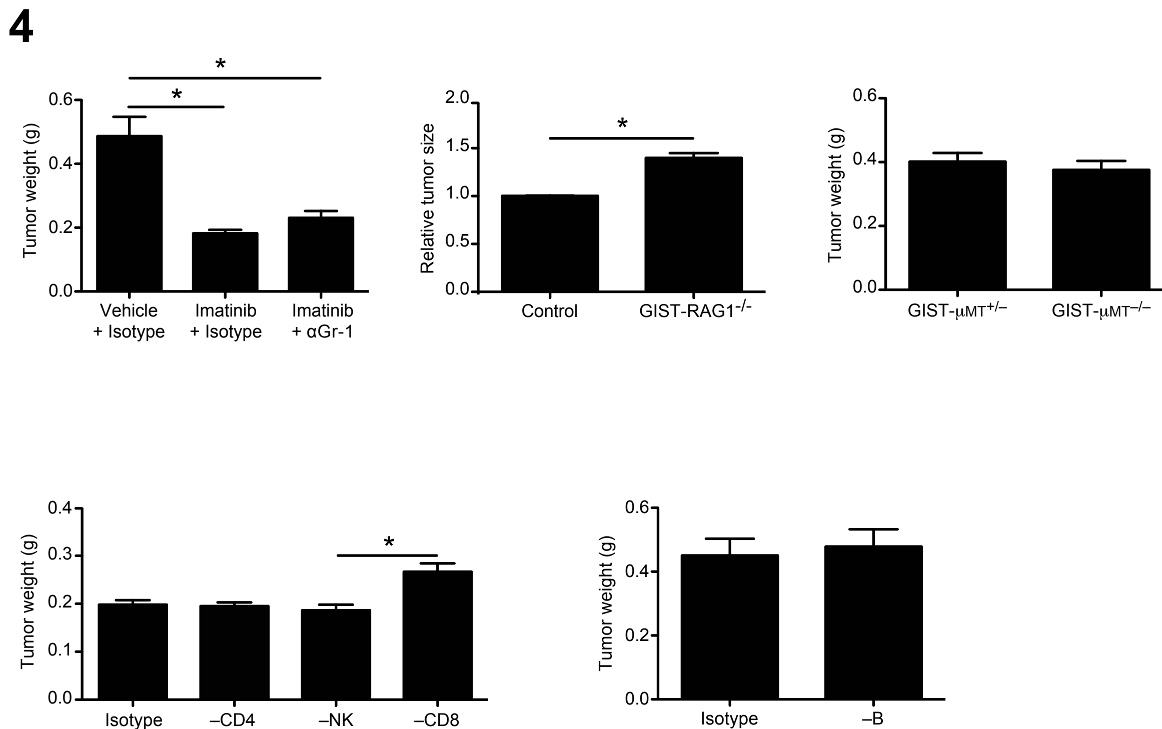
2



After 7 days of treatment with either vehicle or imatinib, we stimulated intratumoral T cells for 4 hours with phorbol 12-myristate 13-acetate and ionomycin and performed intracellular cytokine analysis for IL-4, IL-17, and IFN-γ production. Data represent means ± s.e.m. and are a composite of 2 experiments, each with 3 mice per group.

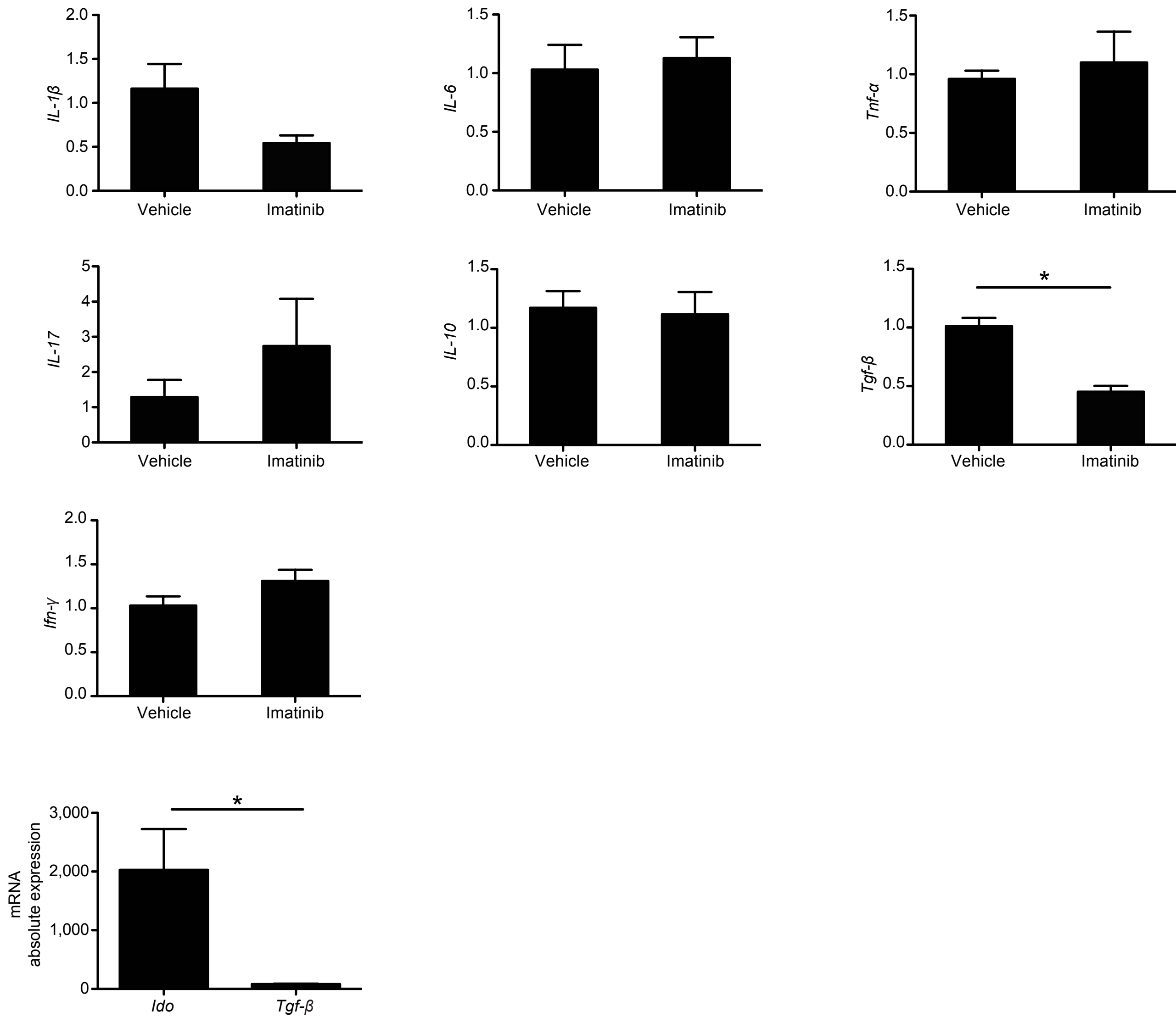


Flow cytometric analysis of tumors after 7 days of treatment with vehicle or imatinib. Myeloid cells were defined as $CD11b^{+}Gr\text{-}1^{+}$. B cells were defined as $CD3^{-}CD19^{+}$. NK cells were defined as $CD3^{-}NK1.1^{+}$. NKT cells were defined as $CD3^{+}NK1.1^{+}$. Numbers indicate frequency of corresponding cells as a percentage of $CD45^{+}$ cells. Data represent means ± s.e.m. n = 7–8 per group.



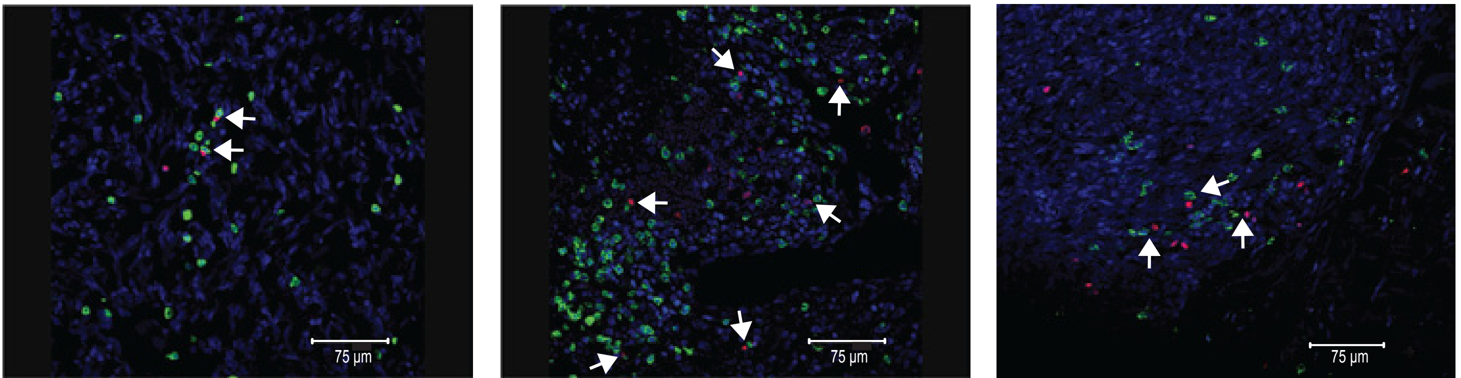
Tumor weight in GIST mice treated with vehicle or imatinib for one week with concurrent depletion of myeloid cells using Gr-1-specific antibody is shown (top left panel, n = 10–13 per group). Tumors were measured in GIST-RAG1$^{-/-}$ mice by either MRI (n = 2) or tumor weight (n = 2) and compared to tumors measured by the same modality in 4 age-matched controls per each GIST-RAG1$^{-/-}$ mouse (n = 16, top middle panel). The significance testing of the ratios was performed using a one-sample t-test after a logarithmic transformation. Tumor weight is shown in GIST-µMT$^{-/-}$ mice that are devoid of B cells compared to age-matched littermate controls (n = 22–27 per group, top right panel). 4 week old GIST mice were depleted of $CD4^{+}$, $NK1.1^{+}$, or $CD8^{+}$ T cells (bottom left) or B cells (bottom right) and tumor weights were analyzed 4 weeks later. Data represent means ± s.e.m. with n = 7–9 per group. * $P < 0.05$.

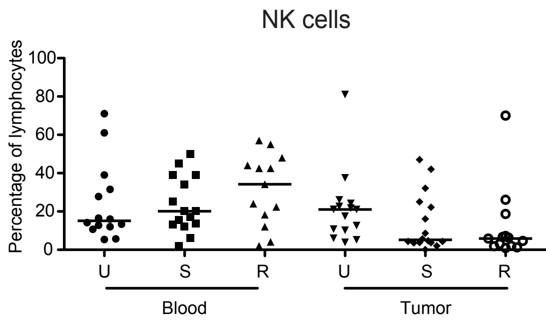
5



RT-PCR of the indicated cytokines in murine GIST tumors after 7 days of treatment with vehicle or imatinib is indicated relative to internal controls (top panels, $n = 6$ per group). *Ido* and *Tgf-β* mRNA expression relative to internal controls in GIST tumors as determined by microarray analysis (bottom panel, $n = 3$ per group). Data represent means ± s.e.m. * $P < 0.05$.

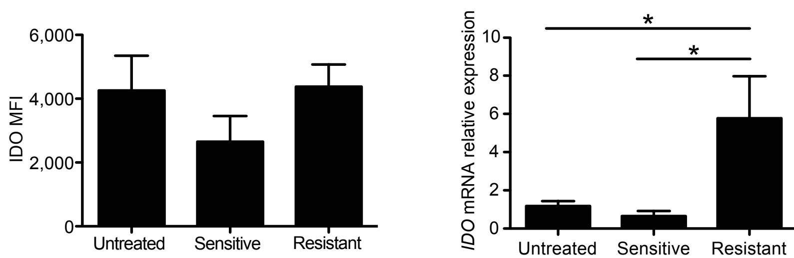
6





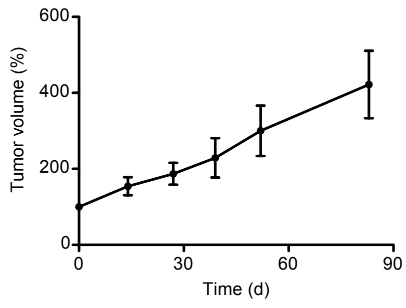
Immunofluorescence demonstrating CD8 (green), FoxP3 (red), and DAPI (blue) staining in human GIST tumors (top panel). Representative staining of 15 specimens analyzed. White arrows indicate co-localization of $CD8^+$ and $FoxP3^+$ cells. Flow cytometric analysis of NK cells in untreated (U), sensitive (S), and resistant (R) GIST specimens (bottom panel).

7



Flow cytometric analysis of IDO expression on tumors cells in untreated, sensitive, and resistant GIST specimens (left panel, n = 3–6 per group); P = 0.17, two-tailed student t-test. *IDO* mRNA in untreated, sensitive, and resistant human GIST specimens (right panel, n = 7–9 per group). Data represent means ± s.e.m. with n as indicated. * P < 0.05.

8



GIST mice were injected with vehicle for one week and serially imaged using MRI. n = 8.

SUPPLEMENTARY TABLE 1

Intratumoral mRNA expression after 1 week of vehicle or imatinib therapy. There were 3 mice per group that were analyzed individually. Only genes with a fold change of at least 2 are listed. Note that the gene symbol for *Ido* is Indo.

Gene Symbol	p-value	Fold-Change	Mean Expression Vehicle	Mean Expression Imatinib
Cxcl9	1.4E–04	–20.0	13,315	964
Cxcl10	7.2E–05	–16.5	4,312	380
1700001L23Rik	2.4E–08	–13.6	916	69
U46068	2.4E–06	–11.0	250	605
Indo	1.7E–04	–6.7	2,028	306
Ngfr	1.9E–07	–6.5	672	102
Etv4	2.7E–05	–5.8	477	88
Htr5b	5.6E–05	–5.8	341	53
Colec11	7.5E–06	–5.3	633	124
Scyb11	8.1E–05	–4.8	44	33
LOC194292	5.1E–06	–4.7	1,161	626
Thrsp	8.8E–06	–4.4	232	100
Slc2a6	6.8E–06	–4.2	859	349
B430320C24Rik	2.2E–04	–4.1	2,419	639
Gpbar1	5.4E–05	–4.1	268	63
Pbk	6.4E–05	–4.1	1,226	291
LOC386475	8.2E–06	–3.9	159	78
Olfm4	1.3E–06	–3.9	189	49
Fetub	2.7E–04	–3.9	839	221
A430056A10Rik	1.6E–05	–3.9	1,167	320
Npr3	8.8E–05	–3.8	563	156
D14Ertd668e	2.4E–04	–3.6	2,016	608
Serpina3g	4.8E–05	–3.5	311	73
Ckap2	1.2E–06	–3.4	179	52
Slc16a4	1.7E–05	–3.4	133	78
1700016C15Rik	3.8E–05	–3.2	287	88
A930031L14Rik	6.4E–06	–3.1	175	56
Pcolce	2.8E–04	–3.1	2,373	760
2310016F22Rik	1.2E–04	–3.1	610	206
Pcdh12	2.3E–05	–3.1	256	83
LOC380706	1.4E–04	–3.1	1,054	361
Pde4c	1.2E–04	–3.0	160	54
Lgals9	9.8E–05	–3.0	105	62
Mx1	4.4E–05	–3.0	190	65
6030405A18	1.1E–05	–2.9	128	43
Irf7	3.9E–04	–2.9	1,741	617

Prc1	2.5E–04	–2.9	1,013	344
9130019I15Rik	3.7E–05	–2.9	968	355
Hpcal4	2.7E–08	–2.9	260	92
H2–T22	1.9E–04	–2.8	1,008	363
D11Lgp2e	1.8E–06	–2.8	634	229
LOC219228	2.4E–05	–2.8	100	230
Tnfrsf21	8.4E–06	–2.8	67	214
Pacrg	1.4E–05	–2.7	159	58
Cd40	6.9E–06	–2.7	868	320
2610318C08Rik	6.5E–05	–2.7	342	124
Dlm1–pending	1.9E–05	–2.7	129	49
Ifi204	4.6E–05	–2.7	246	93
LOC384158	6.3E–05	–2.7	158	420
Bok	9.8E–05	–2.7	1,371	518
Olfr550	2.3E–05	–2.6	261	100
2510004L01Rik	4.0E–04	–2.6	1,945	773
Lgals3bp	2.0E–04	–2.6	399	172
1110030C22Rik	3.5E–04	–2.5	213	84
Ly6e	4.3E–05	–2.5	9,734	3,893
Tpx2	2.9E–04	–2.5	993	577
Gatm	7.1E–05	–2.5	237	97
Slc15a3	5.8E–05	–2.5	96	48
D930046M13Rik	3.5E–05	–2.5	227	91
Cenpa	1.6E–04	–2.5	2,089	841
Ccnb1	1.9E–04	–2.5	497	201
Pcdh17	7.4E–05	–2.4	5,713	2,342
6030405A18Rik	1.9E–04	–2.4	94	39
Gm885	1.1E–04	–2.4	382	160
LOC432555	1.1E–04	–2.4	151	78
Crim1	2.8E–04	–2.4	832	351
E030034P13Rik	2.2E–05	–2.4	410	172
Calmbp1	2.5E–04	–2.4	352	146
1190002F15Rik	3.6E–05	–2.3	272	115
Tapbp	1.1E–04	–2.3	52	77
1200008O12Rik	1.3E–04	–2.3	319	137
H2–T9	8.7E–05	–2.3	7,120	3,161
Hspa12a	2.9E–04	–2.3	325	142
AI505034	2.8E–04	–2.2	83	144
C1s	3.0E–04	–2.2	242	113
Ntrk2	3.3E–04	–2.2	158	70
Rerg	4.1E–04	–2.2	1,800	813
1200009O22Rik	3.6E–04	–2.2	1,768	833
Gdf15	2.6E–04	–2.2	135	61
H2–T10	3.8E–04	–2.1	2,304	1,100
Cdc2a	1.7E–04	–2.1	237	110
Tlr3	2.1E–04	–2.1	89	69
G431001E03Rik	2.1E–05	–2.1	655	311
LOC380732	2.5E–04	–2.1	241	50
KIF23	1.7E–04	–2.1	62	98

Sln	5.8E–05	–2.1	2,436	582
6720460F02Rik	2.7E–04	–2.1	94	45
LOC381319	9.1E–05	–2.1	2,234	768
Ly6a	2.8E–04	–2.1	20,943	10,365
Grp	3.5E–05	–2.1	169	82
Mest	3.3E–05	–2.1	202	97
Pak3	2.0E–04	–2.1	158	76
Dcbld2	2.3E–04	–2.0	197	97
4921511I23Rik	1.1E–04	–2.0	101	49
H2–D4	3.5E–04	–2.0	179	89
Tnfsf10	2.0E–04	–2.0	112	65
A630038D02	1.1E–04	–2.0	309	154
D030038A19Rik	2.9E–04	–2.0	120	61
Sh3md4	2.5E–04	–2.0	4,947	1,424
4732435N03Rik	1.8E–04	2.0	66	132
Syt1	1.8E–04	2.0	149	264
Gng13	2.3E–04	2.0	63	128
Sox10	3.6E–05	2.1	143	314
Ctsf	4.9E–06	2.1	126	263
Plekhb1	3.7E–04	2.1	227	494
Gm1012	5.1E–06	2.2	78	168
Stmn2	2.8E–04	2.2	42	64
Sncg	1.3E–04	2.2	139	249
Cd79a	3.2E–04	2.2	63	140
E130013N09Rik	3.4E–04	2.2	108	239
8430408G22Rik	9.6E–05	2.3	99	225
Ldb2	2.6E–05	2.3	245	61
Edn1	1.6E–04	2.3	190	449
Tsrc1	1.6E–04	2.4	433	170
Pdgfra	1.9E–04	2.4	305	713
Ccl20	1.3E–04	2.5	74	188
Cxcl5	3.4E–04	2.5	48	120
Col23a1	1.4E–04	2.6	148	382
LOC381283	9.1E–05	2.6	122	77
Pou2af1	3.4E–04	2.6	118	300
Per1	3.1E–04	2.7	267	713
BC018285	2.1E–04	2.7	797	2,069
Chst8	4.6E–05	2.8	87	235
Aqp1	2.3E–04	2.8	855	1,294
Mt2	1.0E–04	3.0	134	394
Cldn5	2.7E–05	3.0	91	265
Apod	2.4E–04	3.0	130	86
Hsd11b2	3.7E–04	3.2	134	439
Tnfrsf13c	3.3E–06	3.2	172	116
Ppp1r3c	9.5E–05	3.6	274	995
Cdkn1c	6.4E–07	3.8	483	1,807
Plf2	1.8E–04	4.1	36	162
Cyp2d9	3.9E–07	4.9	90	438

SUPPLEMENTARY TABLE 2

Clinicopathologic characteristics of 45 specimens in 36 patients.

	Untreated	**Sensitive**	**Resistant**
Number of patients	15	13	11[a]
Number of specimens	15	17	13
Median age (range)	68 (41–87)	56 (46–73)	58 (29–75)
Female Male	6 9	2 11	3 8
Primary Metastatic	14 1	8 9	3 10
Primary location Stomach Small intestine Other	 14 1 0	 2 11 0	 5 5 1
Tyrosine kinase inhibitor Imatinib Sunitinib Dasatanib Sorafenib	 Not applicable[b]	 13 2[c] 0 1[c]	 11 3[c] 1[c] 1[c]
Mutational status *KIT* Exon 9 *KIT* Exon 11 *PDGFRA* WT Unavailable tissue	 0 6 3 1 5	 5 6 1 1 4	 2 8[d] 3[e] 0 0
Median treatment (yrs)	Not applicable[b]	0.7 (0.4–3)	2 (0.6–8.9)

[a]3 patients also had sensitive tumors.
[b]1 patient was immediately intolerant to imatinib and sunitinib when treated several years earlier.
[c]Previously failed imatinib.
[d]4 tumors also had secondary mutations in *KIT* exon 13 (V654A) or *KIT* exon 17 (D820H in 2 patients and D820V in 1 patient).
[e]1 patient had a D842V mutation.

SUPPLEMENTARY METHODS

Animals and treatment. Mice were sacrificed at the indicated time points and organs were removed. After weighing the tumors, we mechanically dissociated tumors and DLNs using the flat portion of a plunger from a 3-ml syringe. We then passed the cell suspension through a sterile 100-μm strainer (Falcon; BD Biosciences) followed by an additional 40-μm strainer (Falcon; BD Biosciences) prior to flow cytometric analysis. Depletion of myeloid cells was achieved by i.p. injection of 500 μg Gr-1-specific antibody (clone RB6-8C5, Monoclonal Antibody Core Facility, Sloan-Kettering Institute) on days 0, 1, 4, and 6 of imatinib treatment. Long term B cell depletion was achieved by i.v. injections of 250 μg of CD20-specific antibody (clone 18B12, Biogen Idec) every 2 weeks.

Antibodies. Mouse-specific antibodies to CD8 (clone 53-6.7, AF700), CD4 (clone GK 1.5, APC-Cy7), FoxP3 (clone FJK-16S, APC), granzyme B (clone 16G6, FITC), CD25 (clone PC 61.5, PerCP-Cy5.5), NK1.1 (clone PK136, FITC), and IL-17 (clone eBio17B7, APC) were purchased from eBioscience. Antibodies to Ki67 (clone B56, FITC), CD3 (clone 145-2C11, PerCP-Cy5.5), CD69 (clone H1.2F3, PE), KIT (clone 2B8, APC), Annexin V FITC, IFN-γ (clone XMG1.2, FITC), and IL-4 (clone BVD4-1D11, PE) were obtained from BD Biosciences. Human-specific antibodies included CD3 (clone HIT3a, PE), CD4 (clone RPA-T4, AF700), CD25 (clone M-A251, APC), and CD69 (clone FN50, APC-Cy7) from BD Biosciences and CD8 (clone RPA-T8, PerCP-Cy5.5) from eBioscience. Intracellular cytokine analysis was performed after stimulating tumor infiltrating lymphocytes for 4 h in vitro with 50 ng ml^{-1} phorbol 12-myristate 13-acetate and 500 ng ml^{-1} ionomycin in the presence of Brefeldin A (1 μL per 10^6 cells, BD Biosciences). After restimulation, tumor infiltrating lymphocytes were stained for intracellular cytokines using BD Cytofix/Cytoperm kit according to manufacturer's instructions (BD Biosciences). Antibodies to KIT, STAT3, and S6 were purchased from Cell Signaling. The antibody to ETV4 was purchased

from Santa Cruz Biotechnology. IDO was detected with flow cytometry and western blot using mouse/human-specific IDO (clone 10.1, Millipore). Rat mouse-specific IgG FITC (eBioscience) was used as a secondary antibody for flow cytometry and HRP-conjugated goat antibody specific to mouse IgG for WB (Santa Cruz). IDO staining was classified as hi or low based on the median IDO MFI of the 13 patient sample set. Mouse/human-specific GAPDH (clone 14C10) was purchased from Cell Signaling. Human intracellular FoxP3 expression was assessed using the FITC anti-human FoxP3 Staining Kit (eBioscience). All other intracellular staining was performed using the FoxP3 fixation/permeabilization buffer (eBioscience) according to the manufacturer's instructions.

Immunohistochemistry. Mouse tumors were excised and immediately fixed in 10% formalin, embedded in paraffin, and cut to 5 μm thickness. Hematoxylin and eosin and CD8-specific (Dako) staining was performed using standard methods. Slides were assessed on an Axioplan 2 wide-field microscope (Zeiss).

Immunofluorescence. The indirect, double sequential immunofluorescence detection of CD8 and FoxP3 was performed using Discovery XT processor (Ventana Medical Systems). The tissue sections were blocked for 30 min in mouse IgG blocking reagent (Vector Labs), followed by incubation for 4 h with 5 μg ml^{-1} of the primary antibody (monoclonal mouse antibody specific for human CD8 - Dako clone C8/144B or monoclonal mouse antibody specific for human FoxP3 - Abcam clone 236A/E7) followed by incubation for 60 min with biotinylated mouse-specific IgG reagent (Vector Labs, 1:250 dilution). For detection of CD8 or FoxP3, the tyramide signal amplification kit was used according to the manufacturer’s specifications (Alexa Fluor 488 or Alexa Fluor 568, respectively; Invitrogen).

***Microarray*.** We harvested organs from GIST mice and isolated total RNA using Trizol. Quality of RNA was ensured before labeling by analyzing 20–50 ng of each sample using the RNA 6,000 NanoAssay and a Bioanalyzer 2,100 (Agilent). Samples with a 28S/18S ribosomal peak ratio of 1.8–2.0 were considered suitable for labeling. For samples meeting this standard, 200 ng of total RNA were labeled using the Illumina TotalPrep RNA Amplification kit (Ambion), according to manufacturer's instructions. 3 μg of labeled and fragmented cRNA were then hybridized to the mouse-ref8 array (Illumina), which interrogates ~22,000 transcripts of known genes. Statistical analysis was performed in Partek Genomics Suite version 6.5. Data were log transformed and quantile normalized. ANOVA was performed for the respective comparison groups. Statistically significant genes had a False Discovery Cutoff Rate of 0.05. Genes were further filtered based on fold change of greater or less than 2. The Gene Expression Omnibus accession number for these data is GSE30416.

Western Blotting and Immunoprecipitation. Whole protein was extracted from frozen GIST tissues or from T1 cells using lysis buffer and complete protease inhibitor cocktail (Roche Diagnostics). Protein concentration was determined using the Bradford method. Western blotting was performed using standard methods. For IDO immunoprecipitation in T1 cells, equal amount of cell lysates (150 μg) were incubated with 1 μg of mouse antibody specific to human IDO or control IgG and protein A/G agarose beads for overnight at 4 °C. The supernatants were discarded. Beads were washed with lysis buffer and subjected to SDS-PAGE. The gels were transferred to nitrocellulose and blotted using human-specific IDO IgG (Millipore).

Chromatin Immunoprecipitation (ChIP). ChIP was performed using the ChIP-IT kit (Active Motif, USA) according to the manufacturer's protocol. Briefly, 100 mg GIST tissue was cut into small pieces and cross-linked with 1% formaldehyde in PBS for 15 min at room temperature. Cross-linking was stopped by addition of 0.125 M glycine for 5 min at room temperature. The

tissue was briefly washed with cold PBS and the tissue pieces were minced using a tissue disintegrator for at least 3 min. The homogenized cell suspension was centrifuged at 2,500 rpm at 4 °C for 10 min. The cells were lysed in 1X lysis buffer provided by the manufacturer. The nuclear pellet was obtained by centrifuging the lysed cells at 5,000 rpm for 10 min at 4 °C. Sonication was performed for 15 pulses, with each pulse consisting of 30 s shearing and 20 s placement in ice. The sonicated DNA was checked for sonication efficiency (200–1,500 bp) by running the chromatin on an agarose gel. The chromatin was initially pre-cleared using protein-G beads and subsequently treated with 3 µg Etv4-specific antibody for immunoprecipitation overnight. The immunoprecipitated ChIP product was washed, reverse cross-linked, treated with proteinase K, and eluted to get ChIP-enriched DNA. Simultaneously, control IgG was used as a negative control to check the efficiency of the ChIP experiment. One-tenth of the total lysates was used for total genomic DNA as "input DNA" control. 50 ng of ChIP enriched DNA was used to perform PCR for the *Ido* promoter using the following primers: forward primer (5'-CCAGGATATGTGTTGTTCGTGT-3'), reverse primer (5'-AAAGAGGGGGAGATTCCACC-3'). The PCR products were run on a 2% agarose gel and the amplicons were visualized using UV light.