Critical Role for Kit-mediated Src Kinase But Not PI 3-Kinase Signaling in Pro T and Pro B Cell Development

Valter Agosti, Selim Corbacioglu, Imke Ehlers, Claudia Waskow, Gunhild Sommer, Georgina Berrozpe, Holger Kissel, Christine M. Tucker, Katia Manova, Malcolm A. S. Moore, Hans-Reimer Rodewald, and Peter Besmer

1Developmental Biology and 2Cell Biology Programs, Sloan-Kettering Institute, 3Cornell University Graduate School of Medical Sciences, New York, NY 10021
4Department for Immunology, University of Ulm, D-89070 Ulm, Germany

Abstract

The Kit receptor functions in hematopoiesis, lymphocyte development, gastrointestinal tract motility, melanogenesis, and gametogenesis. To investigate the roles of different Kit signaling pathways in vivo, we have generated knock-in mice in which docking sites for PI 3-kinase (Kit Y719) or Src kinase (Kit Y567) have been mutated. Whereas steady-state hematopoiesis is normal in Kit Y719F/Y719F and Kit Y567F/Y567F mice, lymphopoiesis is affected differentially. The Kit Y567F mutation, but not the Kit Y719F mutation, blocks pro T cell and pro B cell development in an age-dependent manner. Thus, the Src family kinase, but not the PI 3-kinase docking site in Kit, mediates a critical signal for lymphocyte development. In agreement with these results, treatment of normal mice with the Kit tyrosine kinase inhibitor imatinib (Gleevec®) leads to deficits in pro T and pro B cell development, similar to those seen in Kit Y567F/Y567F and Kit W/W mice. The two mutations do not affect embryonic gametogenesis but the Kit Y719F mutation blocks spermatogenesis at the spermatogonial stages and in contrast the Kit Y567F mutation does not affect this process. Therefore, Kit-mediated PI 3-kinase signaling and Src kinase family signaling is highly specific for different cellular contexts in vivo.

Key words: Kit receptor signaling • Src kinase • PI 3-kinase • pro T and pro B cell development

Introduction

Kit encodes a growth factor receptor with ligand-dependent tyrosine kinase activity (1–3). Kit ligand, KitL, is the only known ligand of the Kit receptor (4). The Kit and KitL genes are encoded at the White spotting (W) and Steel (Sl) loci in the mouse, respectively, and loss of function mutations in the Kit and KitL genes generate deficiencies in several cell systems during embryonic development and in the postnatal organism: in hematopoiesis, the pigmentary system, intestinal pacemaker cells, and in gametogenesis. In hematopoiesis, Kit receptor signaling is critical in the stem cell hierarchy, in erythropoiesis, in mast cell development and function, and megakaryopoiesis (5–8). In adult lymphopoiesis, in an age-dependent fashion, Kit has a critical role in pro T and pro B cell subsets (9). In the gastrointestinal tract, Kit signaling is required in interstitial cells of Cajal (ICC), which function as pacemaker cells and mediate inputs from the enteric nervous system to smooth muscle cells (10–12).

KitL binding to the receptor mediates receptor dimerization, activation of kinase activity, and autophosphorylation. Kit activates several signaling cascades leading to cell proliferation, cell survival and other cellular responses. The signaling cascades that are activated by Kit signaling include activation of PI 3-kinase, Src kinases, tyrosine phosphatases, STATs, RAS, RAC, PLC-γ, and CBL. As part of the activation of some of these cascades, signaling molecules containing SH2 domains bind to tyrosine phosphate residues on the activated receptor tyrosine kinase. In the Kit receptor, tyrosines 567, 569, and 719 are known docking sites for src kinases, tyrosine phosphatases, and the p85 subunit of PI 3-kinase, respectively. In vitro studies showed that Kit tyrosine-567 and 719–
phenylalanine substitution mutations block docking and activation of Src family kinase and PI 3-kinase signaling, respectively (13, 14). Furthermore, the Kit tyrosine-567–phenylalanine substitution mutation blocks docking of the tyrosine phosphatase SHP-2 (15), the adapter protein APS, and the Csk-homologous kinase (CHK; reference 16).

Studies in BM-derived mast cells (BMMC) have provided insight about the mechanism by which Kit mediates various cellular responses including cell proliferation, survival, adhesion, actin reorganization, membrane ruffling, and secretion. In BMMC, mutation of the Kit receptor binding sites for class IA PI 3-kinase adaptor proteins, KitY719, and for Src, Kit Y567, were shown to affect cell proliferation, survival, adhesion, and secretion to differing degrees (13, 14, 17). Whereas Kit-mediated PI 3-kinase activation contributes to the mitogenic and survival responses in BMMC, in the secretory response, cell adhesion response, actin polymerization, and membrane ruffling responses, Kit-mediated PI 3-kinase activation is critical. Therefore, divergent downstream signaling pathways are responsible for the generation of various cellular responses.

Diverse Kit receptor functions in vivo may be determined by unique cell type–specific signaling networks. To investigate the contribution of different signaling pathways to various Kit receptor–mediated cellular responses in vivo, we and others have previously generated mice in which the PI 3-kinase binding site KitY719 was mutated (18, 19). The KitY719F mutation was found to affect Kit function only in specific developmental processes, and this is in contrast to other mutations in the Kit receptor gene, i.e., W, which broadly affect Kit function in hematopoiesis, gametogenesis, and melanogenesis. Although the mutation fails to affect steady-state hematopoiesis and skin mast cells, peritoneal mast cell numbers are reduced significantly. Furthermore, although Kit has important functions at multiple stages in embryonic and postnatal gametogenesis, PI 3-kinase signaling is critical only in a specific subset of the postnatal stages in the ovary and testis. We now have generated mice in which the Src binding site in the Kit receptor gene, KitY567, was mutated. Here, we compared the phenotypes of KitY567F mice with the phenotypes of KitY719F mice that display remarkable differences within hematopoiesis as well as germ cell development.

Materials and Methods

Generation of Mutant KitY567F Mice

Site-directed mutagenesis was performed on a 2.4-kb SpeI-KpnI genomic Kit fragment including exons 10–13, mutating tyrosine 567 to phenylalanine in exon 11. The mutant Kit genomic fragment was inserted into a fragment including Kit exons 8–13. A neomycin-resistance gene expression cassette flanked by loxP sites was inserted into a SnaBl site in intron 9. For negative selection a diptheria toxin A (DT-A) gene cassette, provided by Frank Costantini (Columbia University, New York, NY), was placed at the 3’ end of the targeting construct. 129/SvJ embryonic stem (ES) cells (CJ7; Swiatek and Gridley, 1993) were electroporated with linearized targeting construct following standard protocols. Neo-

Animals

C57BL/6J mice were purchased from the JacksonImmunoResearch Laboratories. The KitY719F/KitY719F mice were described previously (18). KitY719F/KitY719F and KitY567F/KitY567F mice used for experiments were backcrossed six times (N6) and three times (N3), respectively, to C57BL/6J mice. Littermates were used as control. Double mutant KitY719F/KitY719F γ−/− mice were generated by crossing KitY719F/KitY719F mice with γ−/− mice (21) as described for KitW/W γ−/− (22).

Mast Cell Cultures

BMMCs from KitY567F/KitY567F, KitY719F/KitY719F, and control mice were derived and cultured as published (14). Kit cell surface expression was monitored by FACS® analysis (see Flow Cytometry Analysis below). Before stimulation, BMMC were washed free of growth factor and cultured in serum-free medium (Stemspan SF expansion medium; StemCell Technologies, Inc.) for 6 h.

Cell Proliferation and Apoptosis

Proliferation assays were performed as described previously (23). In brief, 105 starved BMMC were seeded at 0.2 ml/well in triplicate in 96-well plates, followed by stimulation with KitL (Peprotech) for 24 h. After 20 h, 0.5 μCi of [3H]thymidine was added for 4 h. Cells were harvested and β-emission determined. For apoptosis assays, cells were grown in serum-free media supplemented with IL-3 (20 ng/ml) for 12 h, plated at 106 cells per 2 ml/well in six-well plates, starved for 1 h, and stimulated with KitL at the indicated concentrations for 50 h. Cells were harvested and analyzed by flow cytometry using the Annexin V–FITC detection Kit I (BD Biosciences).

Immunoprecipitation and Western Blotting

Starved BMMC were stimulated with KitL (100 ng/ml) or not for 5 min (Kit IP) or 10 min (Lyn IP and Western blot) at 37°C. Cells were lysed in NP-40 lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 20 μl/ml protease inhibitor cocktail (Sigma–Aldrich). Cleared lysates were precipitated overnight using anti-Kit or anti-Lyn an-
tibody (Santa Cruz Biotechnology, Inc.) and fractionated by SDS-PAGE. For Western blotting, anti-Kit (Oncogene Research Products), anti-Lyn and anti-Actin (Santa Cruz Biotechnology, Inc.), anti–phospho-c–Kit (Tyr 719; Cell Signaling), and anti–PI 3-kinase p85 (Upstate Biotechnology) were used.

Determination of Peripheral Blood Parameters, Mast Cell Numbers, and Histological Analysis

Peripheral blood parameters were analyzed as described previously (25). Skin mast cell numbers were determined as described previously (25). Mast cells per 1-cm skin between the epidermis and the panniculus were counted in several independent sections and averaged. Peritoneal mast cells obtained by lavage of the peritoneal cavity with 5 ml of PBS were cytopsin on slides and stained with Toluidine blue or Alcian blue/Safranin and counted. Berberine sulfate staining for heparin detection was performed according to Enerback (24). 8-μm paraffin sections from Boun’s fixed testis were stained with Hematoxylin-Periodic acid-Schiff according to standard protocol.

Flow Cytometry Analysis

All the monoclonal antibodies used are from BD Biosciences if not differently indicated. Appropriately labeled isotype controls and single/double color–stained cells were always used to define the specific gates. When required, murine Fc block (anti–mouse IgM FITC, or anti-B220 APC/anti-CD43 Cy-Chrome conjugated, and anti–mouse CD25 FITC conjugated) was used. A FACScalibur® (BD Biosciences) was used for analysis.

BMMC Analysis. 3 × 10^5 BMMC resuspended in staining buffer (PBS without Ca^2+ and Mg^2+, 3% FCS, 0.02% NaN₃) were stained for Kit with PE-conjugated anti-Kit antibody and for FceRI by incubation with mouse IgE anti-DNP (clone SPE-7; Sigma-Aldrich) followed by FITC-conjugated IgE monoclonal antibody.

Thymocyte Analysis. Thymi were mechanically dissociated into a single cell suspension, washed, resuspended in staining buffer, and counted. The triple negative (TN) subsets were resolved by staining 1.5 × 10^6 thymocytes with a mix of lineage-specific monoclonal antibodies (anti–mouse Ter119, B220, Mac-1, Gr-1, CD4, CD8, and CD3) PE conjugated, anti–mouse CD44 Cy-Chrome conjugated, and anti–mouse CD25 FITC conjugated. CD44 and CD25 expression was analyzed on the gated lineage negative cells.

B Cell Analysis. Bone marrow was flushed from femurs with PBS, and a single cell suspension was obtained by gentle pipetting and passage through a nylon strainer (Falcon). 1.5 × 10^6 cells resuspended in staining buffer were incubated for 10 min at 4°C with 1 μg of murine Fc block and then labeled with the appropriate monoclonal antibodies mixes: anti–mouse B220 APC/anti-CD43 Cy-Chrome/anti–mouse IgM FITC, or anti-B220 APC/anti-CD43 Cy-Chrome/CD24 FITC/BP-1 PE. Anti-Ki67 PE and anti-CD19 PE were also used in some experiments. When not immediately analyzed the labeled cells were fixed by CytoFix (BD Biosciences) according to the manufacturer’s instruction.

Pharmacological Treatments

STI571 (Gleevec®, Imatinib; Novartis) was administrated intraperitoneally 45 mg/Kg twice daily as shown. At the indicated times animals were killed and thymi and bone marrow were harvested for FACS® analysis.

Statistics

The Student’s t test assuming unequal variances between the two samples was used to determine the significance of differences of lymphocyte subsets between mutants and their littermates. Groups were judged to differ significantly at P < 0.05.

Online Supplemental Material

Fig. S1 shows the pigmentation phenotypes of +/+ , KitY567F/Y567F, KitY719F/Y719F, KitW/+ , KitY567F/W, and KitY719F/W mice. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20031983/DC1.

Results

Point Mutation in the Kit Receptor Gene, KitY567F, Obtained by Gene Targeting Abolishes Lyn Signaling In Vivo. To gain insight into the mechanism of Kit-mediated Src family kinase signaling in vivo, we replaced Kit tyrosine 567 with phenylalanine in the murine Kit gene by using knock-in gene-targeting technology. A targeting construct was made that contained the tyrosine–phenylalanine substitution mutation in Kit exon 11 and a neomycin resistance (neo) cassette flanked by loxP sites for subsequent removal in vivo (Fig. 1 A). Homologous replacement in ES cells produced three correctly targeted ES cell clones identified by PCR, Southern blot, and sequencing analysis. These ES cell clones were microinjected into C57BL/6J blastocysts, chimeras were produced, which gave rise to germline transmission. We have noticed previously that inclusion of a neo-cassette in intronic Kit sequences can interfere with the expression of the Kit gene (18). Therefore, we removed the neo-cassette by cre-mediated excision in vivo as described previously (18). Both heterozygous and homozygous mutant male and female KitY567F/+ and KitY567F/Y567F mice were fertile.

To establish that Kit-mediated Src kinase signaling is abolished in KitY567F/Y567F mice we prepared BMMC from these animals. Mutant BMMC obtained from the KitY567F/Y567F mice have comparable characteristics compared with wild-type BMMC, i.e., expression of cell surface markers, except that Kit receptor levels were reduced to ~50–60% of normal levels (Fig. 1 B). In addition, Kit receptor levels are reduced also in other Kit-expressing cell types such as lineage-negative BM cells (unpublished data). To investigate whether Kit expression was reduced at the RNA or protein level, we perfomed RNase protection assays with RNA from Kit+/+ and KitY567F/Y567F BMMC to determine Kit mRNA levels and found that Kit transcripts are reduced to 50–60% of normal levels (unpublished data). We therefore presume that the remaining lox site and flanking sequences in Kit intron 9 of the mutant affect Kit RNA transcription and/or splicing. It is also possible that the KitY567F mutation affects Kit receptor metabolism/turnover leading to reduced Kit receptor levels. Experiments using mutant BMMC do not support such an explanation, i.e., Kit protein stability upon Kit stimulation of mutant Kit parallels that of wild-type Kit (unpublished data). Because reduced levels of Kit expression may potentially contribute to the phenotype observed in KitY567F/Y567F mice (see below), it is important to note that lymphocyte development is normal in heterozygous KitW/+ mice (9).
To establish that Kit-mediated Lyn signaling is abolished in Kit<sup>Y567F/Y567F</sup> BMMC, we determined whether Lyn and Kit could be coimmunoprecipitated after stimulation of the mutant BMMC with KitL. Cell extracts were immunoprecipitated with anti-Lyn antibody, fractionated by SDS-PAGE, and immunoblotted with anti-Kit and anti-Lyn antibodies. As expected in extracts from wild-type BMMC, Kit could be detected, in BMMC isolated from Kit<sup>Y567F/Y567F</sup> mice association of Lyn with Kit did not occur (Fig. 1 C). However, association of the activated Kit receptor with the p85 subunit of PI 3-kinase was not affected by the Kit<sup>Y567F</sup> mutation. In agreement with this finding Kit tyrosine 719 was phosphorylated comparably in stimulated wild-type and mutant BMMC (Fig. 1 C).

**Steady-state Hematopoiesis and Melanogenesis Are Affected Minimally in Kit<sup>Y567F/Kit<sup>Y567F</sup> and Kit<sup>Y719F/Y719F</sup> Mice.** In hematopoiesis KitL and Kit have multiple roles. In mice carrying W mutations effects are seen in the stem cell compartment, erythropoiesis, and in mast cells (7, 8). Interestingly, in both Kit<sup>Y719F/Y719F</sup> as well as in Kit<sup>Y567F/Y567F</sup> mice hematocrit values and red blood cell, white blood cell, and platelet numbers in mutant mice did not deviate from normal (unpublished data). These results suggest that the Kit-mediated PI 3-kinase signaling and Src kinase family signaling do not have a major role in steady-state hematopoiesis.

Whereas, no pigmentation phenotype was evident in Kit<sup>Y719F/Y719F</sup> mutant mice, hemizygous mutant Kit<sup>Y719F/W</sup> mice displayed a pigmentation phenotype that was stronger than the depigmentation seen in Kit<sup>W/+</sup> mice (Fig. S1). In Kit<sup>Y567F/Y567F</sup> mice ventral depigmentation was seen with variable penetrance and in hemizygous Kit<sup>Y567F/W</sup> this phenotype was enhanced (Fig. S1). Thus, both the Kit<sup>Y567F</sup> and the Kit<sup>Y719F</sup> affect pigmentation, although minimally.

**Age-dependent Effect of the Kit<sup>Y567F</sup> Mutation on T and B Lymphopoiesis.** During fetal and neonatal life, Kit plays a redundant role together with the IL-7 receptor/common cytokine receptor γ chain (γc) in T cell development (22), and no or only a minor role in B cell development (26). Recently, Waskow et al. (9) described an essential, nonredundant role for Kit function in adult lymphopoiesis. A requirement for Kit was found in both pro T and pro B cell subsets, and, interestingly, this requirement was age dependent (9).

To elucidate the molecular mechanism of Kit signaling in lymphopoiesis we investigated the effect of the Kit<sup>Y719F</sup> and Kit<sup>Y567F</sup> mutations on B and T cell lymphopoiesis.

**B Cell Development.** Peripheral blood in Kit<sup>Y719F/Y719F</sup> and in Kit<sup>Y567F/Y567F</sup> mice contained normal numbers of peripheral B lymphocytes (unpublished data). Spleen cellularity in adult mice was not affected in Kit<sup>C<sup>−/−</sup></sup> (1.4 ± 0.52 × 10<sup>8</sup>) and Kit<sup>Y567F/Y567F</sup> (1.2 ± 0.52 × 10<sup>8</sup>) mice, but in Kit<sup>Y719F/Y719F</sup> mice the percentage of splenic B cells (B220<sup>+</sup> cells) was slightly increased (unpublished data). Stages of B cell development in the bone marrow were separated according to Hardy et al. into fractions B (B220<sup>+</sup>CD43<sup>+</sup>CD24<sup>+</sup>BP-1<sup>−</sup>), C (B220<sup>+</sup>CD43<sup>+</sup>CD24<sup>+</sup>BP-1<sup>+</sup>), D (B220<sup>+</sup>CD43<sup>−</sup>IgM<sup>−</sup>), and E (B220<sup>+</sup>CD43<sup>−</sup>IgM<sup>+</sup>) to F (B220<sup>high</sup>CD43<sup>−</sup>IgM<sup>+</sup>; reference 27). Cells in stages B to E represent progressively
increase in percentages of recirculating mature B cells (fraction F; reference 9). In KitY567F/Y567F, but not KitY719F/Y719F mice older than 9 mo, and not in 4-mo-old mice of any genotype, we found a consistent statistically significant reduction of cells in fractions C (B220+CD43+CD24+BP-1-) and D (B220+CD43+/IgM-; Fig. 2 A and Table I). Consistent with these results, the frequencies of Kit+ and CD19+ (a B cell commitment marker) cells within the B220+CD43+CD24hi population (these include cells in fractions B and C) were significantly reduced (Fig. 2 B). The total number of cells in fraction F, mature recirculating B lymphocytes (B220hiCD43IgM+), were not significantly reduced in KitY567F/Y567F when compared with wild-type mice (Fig. 2 A, and Table I). Whereas the block is more complete in KitW/W mice (9) than in KitY567F/Y567F mice, remarkably both mutants display an age-dependent block at the same stage in B cell development.

T Cell Development. First, thymocyte development in KitY719F/Y719F was investigated. Early thymocyte populations were dissected into CD3hiCD4+CD8- (TN stages 1–4; CD44hiCD25- [TN1]; CD44hiCD25+ [TN2]; CD44hiCD25+ [TN3]; CD44+CD25- [TN4]). Comparison of the pro-thymocyte subsets in wild-type and KitY567F/Y567F mice (Fig. 3 A) showed normal proportions in young (unpublished data) as well as in older mice. It was possible that elimination of IL-7Rαγc signaling in pro T cells in KitY719F/Y719F mice would reveal a role for Kit-mediated PI 3-kinase signaling in thymocyte development as mice double null for Kit and γc have no prothymocytes (22, 29). As expected, mice lacking only γc (Kit+/+γc−) had the typical IL-7Rαγc partial block at the TN2 and TN3 to TN4 transition with normal double- and single-positive subsets (for review see reference 28). But analysis of thymocyte development in KitY719F/Y719F γc−/− double-mutant mice revealed a block at the TN3 to TN4 stage as known from IL-7/γc receptor mutants, and no additional arrest due to the KitY719F mutation was observed (Fig. 3 B). Thus, in thymocyte development direct activation of PI 3-kinase by Kit is not required, although in a fetal blood-derived pro T cell line the p85 subunit of PI 3-kinase was recruited to the activated Kit receptor (unpublished data).

Whereas the overall cellularity of thymi in KitY567F/Y567F and control mice was comparable, pro-thymocyte subsets were affected differentially by the mutation (Table II and
Fig. 3 A). FACS analysis of lineage negative thymocytes revealed a clear relative increase in the most immature TN1 (CD44<sup>+</sup>/CD25<sup>-</sup>) subset and a corresponding decrease in the downstream CD25<sup>+</sup> (TN2 and TN3) subset starting at ~6 mo and peaking at ~9 mo in Kit<sup>Y567F/Y567F</sup> mice. At 9 mo the increase of the TN1 subset in Kit<sup>Y567F/Y567F</sup> mice was threefold compared with Kit<sup>/+/+</sup> littermate controls (Fig. 3 C). Whereas the TN1 subset of the lineage negative thymocytes appeared to be increased in mutant mice, when compared with the total thymocyte number the TN1 subset in the mutant mice was un-

Table II. Total Number of T Cell Progenitors for Each Subset in the Thymus of Kit<sup>Y567F/Y567F</sup> and Control Mice

<table>
<thead>
<tr>
<th></th>
<th>Total thymocytes</th>
<th>Lineage negative</th>
<th>TN1</th>
<th>TN2</th>
<th>TN3</th>
<th>TN4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>×10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>×10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;+/+&lt;/sup&gt; (n = 5)</td>
<td>6.36 ± 0.75</td>
<td>1.55 ± 0.17</td>
<td>0.17 ± 0.10</td>
<td>0.08 ± 0.02</td>
<td>1.0 ± 0.21</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;Y567F/Y567F&lt;/sup&gt; (n = 6)</td>
<td>6.67 ± 1.08</td>
<td>0.98 ± 0.23</td>
<td>0.25 ± 0.08</td>
<td>0.04 ± 0.02</td>
<td>0.5 ± 0.19</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>P</td>
<td>0.589</td>
<td>P = 0.001</td>
<td>P = 0.137</td>
<td>P = 0.005</td>
<td>P = 0.003</td>
<td>P = 0.01</td>
</tr>
</tbody>
</table>

Figure 3. Age-dependent block in pro T cell development in Kit<sup>Y567F/Y567F</sup> but not in Kit<sup>/+/+</sup> mice. (A) Expression of CD4 and CD8 was analyzed in 9-mo-old Kit<sup>Y719F/Y719F</sup> and littermate controls (top). Gated lineage negative thymocytes from the same animals were analyzed for CD44 expression to resolve the TN subsets (bottom). (B) Expression of CD4 and CD8 in total thymocytes (top) and of CD44 and CD25 in lineage-negative thymocytes (bottom) was analyzed in Kit<sup>/+/+</sup> and Kit<sup>Y567F/Y567F</sup> mice. (C) CD4 and CD8 expression was analyzed on Kit<sup>Y567F/Y567F</sup> total thymocytes and controls (top) and expression of CD44 and CD25 was analyzed in lineage-negative thymocytes (bottom) from 6- and 9-mo-old Kit<sup>Y567F/Y567F</sup> and control mice.

Figure 4. Age-dependent block in pro T development in C57Bl/6J mice treated with imatinib. (A) Thymus cellularity in 3–6-mo-old C57Bl/6J mice treated with imatinib 45 mg/kg twice daily for 3 or 7 d or with placebo (PBS, 7 d). The cellularity dropped from 7.15 ± 0.8 × 10<sup>7</sup> (placebo, n = 4) to 2.72 ± 0.7 × 10<sup>7</sup> (3 d treatment, n = 6) and 0.46 ± 0.2 × 10<sup>7</sup> (7 d treatment, n = 6). (B) CD4 and CD8 expression analyzed on total thymocytes of 6-mo-old C57Bl/6J mice treated with imatinib for 3 or 7 d or with placebo (top). Gated lineage-negative thymocytes analyzed for CD44 and CD25 expression (bottom). (C) CD4 and CD8 expression analyzed on total thymocytes of 6– or 9-mo-old C57Bl/6J mice treated with imatinib for 7 d or with placebo (9-mo-old mice).
stages (double and single positive) did not seem to be affected. In fact, the overall cellularity of thymi in KitY567F/Y567F mice was normal as well as the fraction of single positive and double positive thymocytes (Fig. 3 C). Moreover, CD4+ and CD8+ lymphocytes in peripheral

Table III. Total Number of B Cell Progenitors for Each Subset in the Bone Marrow of Normal Mice Treated for 7 d with STI571

<table>
<thead>
<tr>
<th></th>
<th>Placebo treated</th>
<th></th>
<th>STI571 treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 4)</td>
<td></td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Cells/femur</td>
<td>3.05 ± 0.5</td>
<td>2.86 ± 0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^7</td>
<td>×10^7</td>
<td>×10^7</td>
</tr>
<tr>
<td>B220+/CD43+</td>
<td>0.99 ± 0.18</td>
<td>0.76 ± 0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^6</td>
<td>×10^6</td>
<td>×10^6</td>
</tr>
<tr>
<td>Fraction A</td>
<td>0.44 ± 0.15</td>
<td>0.41 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^6</td>
<td>×10^6</td>
<td>×10^6</td>
</tr>
<tr>
<td>Fraction B</td>
<td>0.3 ± 0.15</td>
<td>0.25 ± 0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^6</td>
<td>×10^6</td>
<td>×10^6</td>
</tr>
<tr>
<td>Fraction C</td>
<td>0.24 ± 0.07</td>
<td>0.06 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^6</td>
<td>×10^6</td>
<td>×10^6</td>
</tr>
<tr>
<td>B220+/CD43+</td>
<td>4.86 ± 1.58</td>
<td>1.52 ± 0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^6</td>
<td>×10^6</td>
<td>×10^6</td>
</tr>
<tr>
<td>Fraction D</td>
<td>2.2 ± 0.96</td>
<td>0.07 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^6</td>
<td>×10^6</td>
<td>×10^6</td>
</tr>
<tr>
<td>Fraction E</td>
<td>0.69 ± 0.21</td>
<td>0.1 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>×10^6</td>
<td>×10^6</td>
<td>×10^6</td>
</tr>
<tr>
<td>Fraction F</td>
<td>1.82 ± 0.7</td>
<td>1.33 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>P = 0.545</td>
<td>P = 0.07</td>
<td>P = 0.699</td>
<td></td>
</tr>
<tr>
<td>P = 0.01</td>
<td>P = 0.02</td>
<td>P = 0.02</td>
<td></td>
</tr>
<tr>
<td>P = 0.01</td>
<td>0.268</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. Age-dependent block in pro B cell development in C57Bl/6J mice treated with imatinib. (A) B220+/CD43+ BM cells were analyzed for IgM expression (a–d), and B220+/CD43+ cells were analyzed for CD24 and BP-1 expression (e–h) in 3- and 9-mo-old C57Bl/6J mice treated with imatinib 45 mg/kg twice daily for 7 d. (B) Expression of CD24 and Kit (a and b) or CD19 (c and d) was analyzed within the B220+/CD43+ gate in 9-mo-old imatinib-treated and placebo-treated mice.

changed, but the TN2, TN3, and TN4 subsets were reduced by 50% (Table II). Hence, KitY567F/Y567F but not KitY719F/Y719F mice display an age-dependent effect on thymocyte development. However, this developmental retardation was incomplete and differentiation into later
blood, spleen, and bone marrow were also present in normal percentages (unpublished data).

Taken together, these results revealed an age-dependent requirement for Kit-mediated Src kinase family signaling but not PI 3-kinase signaling in early B and T cell development similar to that seen in Kit<sup>W/W</sup> mice. Whereas the developmental arrest is more complete in Kit<sup>W/W</sup> mice (9) than in Kit<sup>Y567F/Y567F</sup> mice, remarkably, both mutants display an age-dependent block at the same stages in B and T cell development.

Pharmacological Inhibition of Early B and T Lymphopoiesis with the Kit Tyrosine Kinase Inhibitor Imatinib (Gleevec®).

Targeted drug therapy for the treatment of malignancies has had a dramatic breakthrough and clinical success with the tyrosine kinase inhibitor imatinib (Gleevec®; Novartis), an inhibitor of the ABL, platelet-derived growth factor receptor (PDGFR), and Kit kinases. Gleevec<sup>®</sup> is successfully used to treat patients with chronic myelogenous leukemia as well as patients with gastrointestinal stromal tumor. Because of its specific effect on Kit signaling we wondered whether pharmacological inhibition of Kit signaling by Gleevec<sup>®</sup> would block normal steady-state lymphocyte development in adult mice, and this was indeed the case. In young adult mice (3–6 mo) imatinib induced a dramatic decrease in thymus size and cellularity after 3 d and more pronounced after 7 d of treatment with the drug (Fig. 4 A). The CD4<sup>+</sup> CD8<sup>+</sup> subset was strongly reduced, and analysis of the TN subsets revealed a partial block at the TN1 stage. The lineage negative CD44<sup>+</sup> CD25<sup>−</sup> thymocytes were increased at the expense of the CD44<sup>+</sup> CD25<sup>+</sup> and the CD44<sup>+</sup> CD25<sup>−</sup> subsets (Fig. 4 B). These effects were even more dramatic upon treatment of older mice (>9 mo). In these older mice the double positive subset was reduced to <10% of the control (Fig. 4 C).

To investigate the effect of imatinib on B cell differentiation we analyzed the bone marrow of mice after 7 d of drug treatment. In young adult mice a reduction of the B220<sup>+</sup> CD43<sup>−</sup> CD24<sup>−</sup> BP-1<sup>−</sup> (fraction C) and B220<sup>+</sup> CD43<sup>+</sup> IgM<sup>−</sup> (fraction D) subsets was observed similar to that seen in Kit<sup>Y567F/Y567F</sup> mice. Again, treatment of older mice produced a stronger phenotype. In these mice fraction C pro B cells were reduced fourfold compared with placebo-treated mice (Fig. 5 A and Table III). Similarly to what was observed in Kit<sup>Y567F/Y567F</sup> mice, the Kit<sup>+</sup> CD19<sup>+</sup> cells within the B220<sup>+</sup> CD43<sup>+</sup> CD24<sup>−</sup> subset were severely depleted in imatinib treated mice (Fig. 5 B). Taken together these results demonstrate that acute inhibition of Kit signaling by imatinib strongly affected mouse lymphopoiesis mimicking the effects seen in mice carrying germline Kit loss of function mutations, Kit<sup>W/W</sup> and Kit<sup>Y567F/Y567F</sup>.
Peritoneal Connective Tissue Mast Cells But Not Skin Connective Tissue Mast Cells Are Affected in Both KitY567F/Y567F and KitY719F/Y719F Mice. KitL and Kit play a critical role in mast cell development and maintenance in the adult animal (7, 8). Interestingly, both the KitY719F and the KitY567F mutation do not affect mast cell numbers in the dorsal skin but peritoneal mast cell numbers are severely diminished in these mice (Table IV). Staining with berberine sulfate and alcian/safranin of peritoneal mast cells indicates that although the mutations affect mast cell numbers they do not affect their development and differentiation. Thus, fully differentiated mast cells were detected, albeit at much reduced numbers, in both KitY567F/Y567F and KitY719F/Y719F mice (Fig. 6 A).

In contrast to this mast cell deficiency in vivo in KitY567F/Y567F mice, KitL-mediated proliferation of KitY567F/Y567F BMMC was markedly enhanced, particularly at low doses of KitL (Fig. 6 B). Most likely this results from the effects of the mutation on the recruitment of the tyrosine phosphatase SHP2 and the adaptor protein APS, which are affected by the Y567F mutation (15, 16). Survival was marginally enhanced in KitY567F/Y567F BMMC.

Differential Effects of the KitY567F and the KitY719F Mutations in Spermatogenesis. KitY567F heterozygous and homozygous mice were viable, healthy, and they were born at Mendelian frequencies. Our previous analysis of gametogenesis in KitY719F/Y719F mice showed lack of an effect of the mutation on the embryonic stages of gametogenesis (18). In contrast, in males spermatogenesis was blocked during the premeiotic spermatogonial stages and oogenesis in females was affected to a lesser degree during the cuboidal stage of follicle development (18). The KitY567F mutation did not affect fertility of homozygous mutant males and females and histological analysis confirmed these findings (Fig. 7). In tubules of adult male mutant tests all stages of spermatogonial maturation and differentiation are seen, although focal areas (<5%) contained empty tubules (Fig. 7). They were found in young, adult, and old mice and the severity was not age related. Therefore, whereas Kit-mediated PI 3-kinase signaling is a critical step in spermatogenesis; in contrast the KitY567F mutation does not affect this process.

### Table IV. Number of Mast Cells in the Skin and Peritoneum of KitY719F/Y719F and KitY567F/Y567F Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>Skin mast cells</th>
<th>Peritoneal mast cells (%) of total peritoneal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KitY719F/Y719F</td>
<td>16–32</td>
<td>223 ± 13 (n = 3)</td>
<td>0.98 ± 0.64 (n = 5)</td>
</tr>
<tr>
<td>KitY567F/Y567F</td>
<td>16–32</td>
<td>199 ± 18 (n = 3)</td>
<td>3.58 ± 1.69 (n = 6)</td>
</tr>
<tr>
<td>KitY719F/Y719F</td>
<td>18–24</td>
<td>153 ± 30 (n = 3)</td>
<td>0.07 ± 0.056 (n = 3)</td>
</tr>
<tr>
<td>KitY567F/Y567F</td>
<td>18–24</td>
<td>150 ± 9 (n = 3)</td>
<td>3.2 ± 0.76 (n = 3)</td>
</tr>
<tr>
<td>KitY719F/Y719F</td>
<td>16–32</td>
<td>199 ± 18 (n = 3)</td>
<td>3.58 ± 1.69 (n = 6)</td>
</tr>
<tr>
<td>KitY567F/Y567F</td>
<td>16–32</td>
<td>223 ± 13 (n = 3)</td>
<td>0.98 ± 0.64 (n = 5)</td>
</tr>
</tbody>
</table>

Values taken from reference 18.

### Discussion

In an attempt to understand the biological roles of RTK-mediated Src family kinase and PI 3-kinase signaling in vivo we have characterized the phenotypes of mice expressing mutant Kit receptors, which fail to activate PI 3-kinase or Src family kinase signaling, KitY719F and KitY567F, obtained by a knock-in strategy. Blockade of either of the two signaling pathways produces phenotypes in distinct cell populations in early B and T cell development, in mast cells, and in spermatogenesis. But in most other cell types, mutant phenotypes are minor. Similar mutational analysis of PDGF receptor α chain signaling in vivo in which the analogous PI 3-kinase and Src binding sites were mutated gave rather dif-
different results (30). On one hand the PI 3-kinase binding site mutation produced severe phenotypes in many cell types. In contrast, the Src binding site mutation had a limited phenotype in oligodendrocyte development. These results would suggest that PDGF-mediated PI 3-kinase signaling is critical for PDGF function in vivo. Therefore, analogous mutations in the closely related Kit and PDGF receptor ζ chain have very different in vivo consequences. This raises the possibility that the cellular context in which the receptor functions may have a very critical role.

Whereas the Y719F mutation blocks the direct binding by Kit of the p85 regulatory subunit of PI 3-kinase and its activation, the Y567F mutation blocks Src kinase family binding and activation as well as binding of the tyrosin phosphatase SHP2 and APS family adaptor proteins (14–16). Therefore, the interpretation of the consequences of the Y567F mutation is quite complex as three different signaling events have to be considered as well as the specific cellular context. The Src family of kinases includes Src, Yes, Lyn, Fyn, Blk, Lck, and Fgr. Whereas, Src and Yes are expressed ubiquitously, the other members are expressed in a tissue-specific manner and they may activate distinct cellular responses (31, 32). Therefore, the Y567F mutation may block activating as well as inhibitory signaling events related to cell proliferation, cell survival as well as receptor desensitization.

Both the Kit Y567F and the KitY719F mutation affect Kit function only in specific developmental processes and this is in contrast to other mutations in the Kit receptor gene that broadly affect Kit function in hematopoiesis, gametogenesis, and melanogenesis. Thus, both mutations fail to affect steady-state hematopoiesis and tissue mast cell numbers, but they substantially reduce peritoneal mast cell numbers. Furthermore, although Kit has important functions at multiple stages in embryonic and postnatal gametogenesis, the KitY719F mutation, and thus PI-3 kinase signaling is critical only in a specific subset of the postnatal stages in the ovary and testis, but the KitY567F mutation does not affect these processes. The KitY719F but not the KitY567F mutation affects activation of the PI 3-kinase Akt signaling cascade known to have a critical role in mediating cell survival (unpublished data), thus during the spermatogonial stages Kit signaling appears to be critical in mediating cell survival (18). We conclude, therefore, that in most cells that require Kit, there are redundant signaling pathways, but that in certain cell types, the PI-3 kinase pathway is critical.

In embryogenesis and in neonatal mice T cell development is reduced, but permissive, and B cell development is unaffected by loss of Kit function. In adult mice, recent studies demonstrated a role for Kit function in both B and T cell development (9). In viable Kit-null mutant mice an age-dependent progressive decline of pro B and pro T cells was observed with a concurrent loss of common lymphoid progenitors in the bone marrow.

In B cell development, the Kit-null mutation, KitWv, does not affect the immunoglobulin repertoire and the establishment of the peripheral B cell compartment, but Kit function is required for the maintenance of B lymphopoiesis from hematopoietic stem cells in adult mice (9). In KitY567F/KitY567F, but not in KitY719F/KitY719F mice, we demonstrate a partial block during the Kit-positive pro B cell stages in agreement with these earlier results. Whereas, hematopoietic stem cells and early progenitor numbers in the BM determined by FACS are normal in both KitY567F/KitY567F and in KitY719F/KitY719F mice (unpublished data), in BM of KitY567F/KitY567F mice there was a progenitor deficit with reduction in fraction C and D, but mature recirculating B lymphocytes were not significantly reduced. It is of interest to note that this phenotype emerges late in life, i.e., it is detectable at 9 mo and becomes more prominent in older mice. Young adult animals (4-mo-old) are not affected. It is possible that an age-related change in the bone marrow microenvironment may contribute to the expression of this phenotype. Thus, signaling mediated by phosphorylation of KitY567F is important for Kit function in pro B cell development in an age-dependent fashion. Identity of the possible Src family kinase members involved in this is not known. In addition, contributory roles of SHP-2 and the APS adaptor are difficult to evaluate as well. However, Kit-mediated activation of PI 3-kinase does not appear to be critical. In contrast to spermatogenesis in lymphocyte development a Kit-mediated survival signal is either not critical or compensated for by another signaling mechanism. The signal provided by Kit-PYS67, which is critical for lymphocyte development, on the other hand may effect either cell proliferation and/or differentiation.

In T cell development loss of Kit function over time produces a progressive loss of pro-thymocytes. There is an age-dependent block at the TN2 stage and consequently accumulation of CD44+CD25−Kit+TN1 cells as well as a reduction of double-positive and single-positive thymocytes (9). In thymi of KitY567F/KitY567F, but not KitY719F/KitY719F mice, analysis of lineage-negative thymocytes showed an increase of TN1 cells and a corresponding decrease of the CD25+TN3 cell populations. But similar to thymi of mice carrying the hypomorphic Kit allele KitWv, single- and double-positive thymocyte numbers in KitY567F/KitY567F thymi were not affected. In T cell development age also plays an important role. A mutant phenotype is first detected at 6 mo and is more severe at 9 mo. Thus, the effect of the KitY567F mutation on thymocyte development is partial and age related. In contrast the KitY719F mutation does not affect thymopoiesis.

Mast cells arise from progenitors in the bone marrow, however, maturation and differentiation of these cells occurs mainly in tissues where they reside (33, 34). Kit has a major role in mast cell development and mast cell function and mice with Kit loss of function mutations lack mast cells in various tissues of the adult organism including skin, mucosa of the GI tract, lung, peritoneal cavity, and its associated mesentery. Interestingly, in both KitY719F/KitY719F and KitY567F/KitY567F mice mast cell numbers in dorsal skin sections were not reduced. In contrast, both mutations affected peritoneal mast cell numbers to differing degrees. Importantly, remaining peritoneal mast cells in the two mutant mice were fully differentiated. Quite likely then the perito-
neal microenvironment may not be able to compensate the Kit signaling deficiencies.

The tyrosine kinase inhibitor STI571/imatinib is a specific inhibitor for the ABL, ARG, PDGFR, and Kit kinases. It is used to treat chronic myelogenous leukemia and gastrointestinal stromal tumor patients with considerable success. An important clinical attribute of this drug is the lack of major known side effects. Since imatinib also inhibits kinases other than Kit, notably ABL, blocking their function may also contribute to the phenotypes in mice treated with imatinib. A role for ABL in B cell development has been inferred from phenotypes of mice with an ABL-null mutation (35, 36). However, we consider this possibility unlikely because (a) the phenotypes in pro B cell development in ABL mutant mice are quite variable and affect all pro B cell subsets, and (b), perhaps more importantly, treatment of mice with imatinib produces similar, age-dependent pro T and pro B cell phenotypes as those seen in mice carrying germine Kit mutations (this paper and reference 9). The overlapping phenotypes caused by genetic and pharmacological modes of Kit inhibition affected the same signaling pathways in vivo. Moreover, similar to mice carrying germine Kit mutations, which lack Kit function throughout life, our data using Imatinib show that acute inhibition of Kit signaling in older mice rapidly affects lymphopoiesis. The increased Kit dependency of lymphopoiesis with age may result from age-dependent changes in the bone marrow and the thymic microenvironment such that Kit signaling becomes more critical. The molecular nature of the age-associated changes remains to be determined. The effect of STI571 on the immune system’s response to an antigen has been assessed in a 4-week study in rats (8-week-old). STI571 was administered orally up to 60 mg/kg/d. Relative counts of lymphocyte subpopulations in peripheral blood, thymus, spleen, and mesenteric lymph nodes revealed no obvious changes attributable to either administration of STI571 or immunization of animals with KLH. There were no findings indicative of an adverse immunotoxicological effect (Paul, G.R., U. Junker, and P. Ulrich, personal communication).

We would like to thank Drs. Elizabeth Lacy and Willie Mark for advice with the gene targeting experiments, Dr. Alexandra Joyner for the pKSloxPNT plasmid, and Drs. Heiner Westphal and Monica Bessler for the EIIa-cre mice. We thank Dr. Jan Hendrix for help with FACS® analysis, Harry Satterwhite for expert assistance with hematological determinations, and Sandra Gonzales, Melissa Agosti et al.877

References
17. Vosseller, K., G. Stella, N.S. Yee, and P. Besmer. 1997. c-kit receptor signaling through its phosphatidylinositol-3’-kinase-binding site and protein kinase C: role in mast cell...


