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

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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–

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Abbreviations used in this paper: BMMC, BM-derived mast cells; CHK, Csk-homologous kinase; DT-A, diphtheria toxin A; ES, embryonic stem; ICC, interstitial cells of Cajal; KitL, Kit ligand; Sl, Steel; TN, triple negative; W, White spotting.

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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 adaptor 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 Kit^{Y719} was mutated (18, 19). The Kit^{Y719F} 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, Kit^{Y567}, was mutated. Here, we compared the phenotypes of $Kit^{Y567F/Y567F}$ with the phenotypes of $Kit^{Y719F/Y719F}$ mice that display remarkable differences within hematopoiesis as well as germ cell development.

Materials and Methods

Generation of Mutant Kit^{Y567F} 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 diphtheria 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. Neomycin-resistant ES cell clones were isolated and analyzed for homologous recombination. First, ES cell clones were screened by a PCR strategy (primer set A/B) in which a 5.0-kb fragment including the neo-cassette and a genomic region outside the target construct was amplified. Correctly targeted ES cell clones then were verified by Southern blot analysis and the presence of the Kit^{Y567F} was confirmed by sequence analysis. Correctly targeted ES cell clones were microinjected into C57BL/6J blastocysts and male mice displaying 85–100% chimerism were backcrossed to C57BL/6J females for germline transmission.

The floxed neo-cassette was excised in vivo by mating heterozygous mutant males with EIIa-cre transgenic females (20). EIIa-cre transgenic mice on a C57BL/6J background (N10) were kindly provided by Monica Bessler (Washington University Medical School, St. Louis, MO). Excision of the neo-cassette was monitored by using DNA digested with BamHI and Southern blot analysis using Kit- and Neo-specific probes. In addition the residual lox site and adjacent multiple cloning sites were identified by PCR (lox-PCR: primer set C/D). Primer A: 5′-AAGAACTCGTCAAGAAGGCGATAGAAGGCG-3′; primer B: 5′-CTCCGTTGAGTGCAGAAGGTTC-3′; primer C: 5′-ACGATGTGGGCAAGAGTT-3′; and primer D: 5′-GATACTGTTAACATTTCGATACAGATGTTTAGC-3′.

Animals

C57Bl/6J mice were purchased from the Jackson ImmunoResearch Laboratories. The $Kit^{Y719F/Y719F}$ mice were described previously (18). $Kit^{Y719F/Y719F}$ and $Kit^{Y567F/Y567F}$ 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 $Kit^{Y719F/Y719F}$ $\gamma_c^{-/-}$ were generated by crossing $Kit^{Y719F/+}$ mice with $\gamma_c^{-/-}$ mice (21) as described for $Kit^{W/W}$ $\gamma_c^{-/-}$ (22).

Mast Cell Cultures

BMMCs from $Kit^{Y567F/Y567F}$, $Kit^{Y719F/Y719F}$, 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, 10^5 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 [^{3}H]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 10^6 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 Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 20 μl/ml proteinase 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 cytospun 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 Bouin'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 CD16/CD32 monoclonal antibody) 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% NaN_3) 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-Kit 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 $^{+/+}$, $Kit^{Y567F/Y567F}$, $Kit^{Y719F/Y719F}$, $Kit^{W/+}$, $Kit^{Y567F/W}$, and $Kit^{Y719F/W}$ mice. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20031983/DC1.

Results

Point Mutation in the Kit Receptor Gene, Kit^{Y567F}, 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 neomycine 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 $Kit^{Y567F/+}$ and $Kit^{Y567F/Y567F}$ mice were fertile.

To establish that Kit-mediated Src kinase signaling is abolished in $Kit^{Y567F/Y567F}$ mice we prepared BMMC from these animals. Mutant BMMC obtained from the $Kit^{Y567F/Y567F}$ 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 $Kit^{Y567F/Y567F}$ BMMC to determine Kit RNA 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 Kit^{Y567F} 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 Kitl 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 $Kit^{Y567F/Y567F}$ mice (see below), it is important to note that lymphocyte development is normal in heterozygous $Kit^{W/+}$ mice (9).

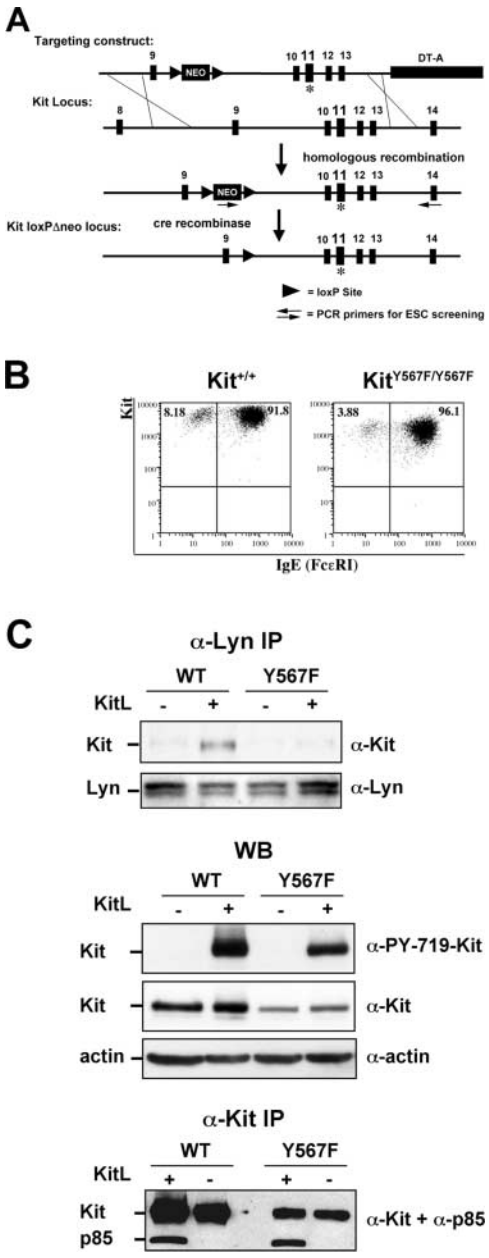

Figure 1. Targeted substitution of Kit tyrosine 567 by phenylalanine in the 129/Sv Kit locus. (A) Schematic representation of targeting strategy: LoxP sites are indicated by rectangles and a red star highlights exon 11. For negative selection a diphteria A gene-cassette (DT-A) was placed at the 3′ end of the construct. (A and B) Characterization of BMMC from $Kit^{Y567F/Y567F}$ mice. (B) Expression of Kit and FcεRI was analyzed by FACS®. (C, top) Coimmunoprecipitation of Kit with Lyn in $Kit^{Y567F/Y567F}$ and WT BMMC treated with KitL. Starved cells were treated with and without KitL (100 ng/ml) for 10 min at 37°C. Cell lysates were immunoprecipitated with anti-Lyn antibody, fractionated by SDS/PAGE, and blotted with anti-Kit antibody and Lyn antibody. (Middle) Western blot of $Kit^{Y567F/Y567F}$ and WT BMMC extracts obtained after stimulation with KitL (10 min), blotted with anti–phospho-Kit (Tyr 719), anti-Kit, and anti-actin antibodies is shown. Coimmunoprecipitation of the p85 subunit of PI 3-kinase with Kit is shown in the bottom panel. Extracts of $Kit^{Y567F/Y567F}$ and WT BMMC treated with KitL (5 min) were immunoprecipitated with anti-Kit antibody, fractionated by SDS-PAGE, and Western blots developed with anti-Kit and anti-p85 antibodies.

To establish that Kit-mediated Lyn signaling is abolished in $Kit^{Y567F/Y567F}$ 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 $Kit^{Y567F/Y567F}$ 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^{Y567F} 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^{Y567F}/Kit^{Y567F} and $Kit^{Y719F/Y719F}$ 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 $Kit^{Y719F/Y719F}$ as well as in $Kit^{Y567F/Y567F}$ 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 $Kit^{Y719F/Y719F}$ mutant mice, hemizygous mutant $Kit^{Y719F/W}$ mice displayed a pigmentation phenotype that was stronger than the depigmentation seen in $Kit^{W/+}$ mice (Fig. S1). In $Kit^{Y567F/Y567F}$ mice ventral depigmentation was seen with variable penetrance and in hemizygous $Kit^{Y567F/W}$ this phenotype was enhanced (Fig. S1). Thus, both the Kit^{Y567F} and the Kit^{Y719F} affect pigmentation, although minimally.

Age-dependent Effect of the Kit^{Y567F} 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^{Y719F} and Kit^{Y567F} mutations on B and T cell lymphopoiesis.

B Cell Development. Peripheral blood in $Kit^{Y719F/Y719F}$ and in $Kit^{Y567F/Y567F}$ mice contained normal numbers of peripheral B lymphocytes (unpublished data). Spleen cellularity in adult mice was not affected in $Kit^{+/+}$ ($1.4 \pm 0.52 \times 10^8$) and $Kit^{Y567F/Y567F}$ ($1.2 \pm 0.52 \times 10^8$) mice, but in $Kit^{Y567F/Y567F}$ mice the percentage of splenic B cells ($B220^+$ 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 ($B220^+CD43^+CD24^+BP\text{-}1^-$), C ($B220^+CD43^+CD24^+BP\text{-}1^+$), D ($B220^+CD43^-IgM^-$), and E ($B220^+CD43^-IgM^+$) to F ($B220^{high}CD43^-IgM^+$; reference 27). Cells in stages B to E represent progressively

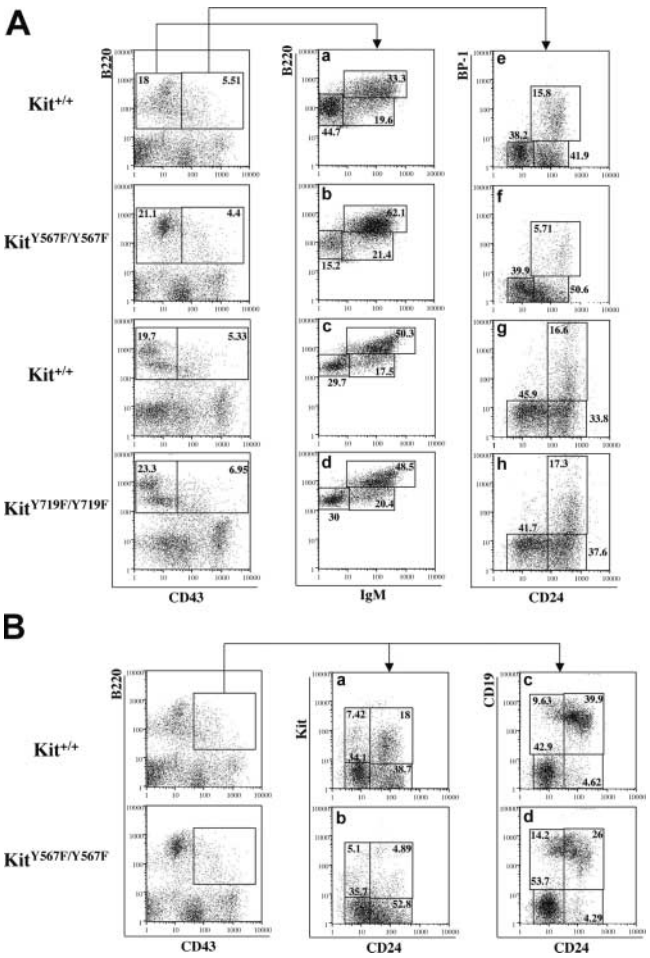

Figure 2. Age-dependent block in pro B cell development in $Kit^{Y567F/Y567F}$, but not in Kit^{Y719F}/Kit^{Y719F} mice. (A) Expression of B220, CD43, CD24, BP-1, and IgM in BM cells of 14-mo-old $Kit^{Y567F/Y567F}$, $Kit^{Y719F/Y719F}$ mutant mice, and respective littermate controls. Stages of B cell development were resolved according to Hardy et al. (27). The $B220^+$ $CD43^-$ cells were analyzed for IgM expression (a–d) and $B220^+$ $CD43^+$ cells were analyzed for CD24 and BP-1 expression (e–h). (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 $Kit^{Y567F/Y567F}$ and control mice.

maturing precursor B cells, and F represents recirculating mature B cells. Previously, an age-dependent block in B cell development beginning at stage B, and most evident from stage C onwards was demonstrated in $Kit^{W/W}$ mice. This progenitor loss was associated with a corresponding relative increase in percentages of recirculating mature B cells (fraction F; reference 9). In $Kit^{Y567F/Y567F}$, but not $Kit^{Y719F/Y719F}$ 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 ($B220^+CD43^+CD24^+BP\text{-}1^+$) 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 $B220^+CD43^+CD24^{high}$ 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 ($B220^{high}CD43^-IgM^+$), were not significantly reduced in $Kit^{Y567F/Y567F}$ when compared with wild-type mice (Fig. 2 A, and Table I). Whereas the block is more complete in $Kit^{W/W}$ mice (9) than in $Kit^{Y567F/Y567F}$ mice, remarkably both mutants display an age-dependent block at the same stage in B cell development.

T Cell Development. First, thymocyte development in $Kit^{Y719F/Y719F}$ mice was investigated. Early thymocyte populations were dissected into $CD3^-CD4^-CD8^-$ (TN stages 1–4: $CD44^+$ $CD25^-$ [TN1]; $CD44^+$ $CD25^+$ [TN2]; $CD44^-$ $CD25^+$ [TN3]; $CD44^-$ $CD25^-$ [TN4]). Comparison of the pro-thymocyte subsets in wild-type and $Kit^{Y719F/Y719F}$ mice (Fig. 3 A) showed normal proportions in young (unpublished data) as well as in older mice. It was possible that elimination of IL-7Rα/γ_c signaling in pro T cells in $Kit^{Y719F/Y719F}$ mice would reveal a role for Kit-mediated PI 3-kinase signaling in thymocyte development as mice double null for Kit and γ_c have no pro-thymocytes (22, 29). As expected, mice lacking only γ_c ($Kit^{+/+}$ γ_c^-) had the typical IL-7Rα/γ_c 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 $Kit^{Y719F/Y719F}$ $\gamma c^{-/-}$ 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 Kit^{Y719F} 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 $Kit^{Y567F/Y567F}$ and control mice was comparable, pro-thymocyte subsets were affected differentially by the mutation (Table II and

Table I. *Total Number of B Cell Progenitors in the Bone Marrow of $Kit^{Y567F/Y567F}$ and Control Mice*

	Cells/femur $\times 10^7$	$B220^+/CD43^+$ $\times 10^6$	Fraction A $\times 10^6$	Fraction B $\times 10^6$	Fraction C $\times 10^6$	$B220^+/CD43^-$ $\times 10^6$	Fraction D $\times 10^6$	Fraction E $\times 10^6$	Fraction F $\times 10^6$
$Kit^{+/+}$ ($n = 4$)	2.91 ± 0.19	1.15 ± 0.16	0.44 ± 0.08	0.48 ± 0.24	0.27 ± 0.03	5.8 ± 0.93	2.82 ± 0.61	0.89 ± 0.06	1.92 ± 0.46
$Kit^{Y567F/Y567F}$ ($n = 5$)	2.68 ± 0.76	0.65 ± 0.18	0.47 ± 0.19	0.16 ± 0.08	0.11 ± 0.03	2.52 ± 0.55	0.67 ± 0.22	0.21 ± 0.08	1.51 ± 0.38
	P = 0.534	P = 0.003	P = 0.782	P = 0.06	P = 0.0002	P = 0.005	P = 0.002	P = 0.001	P = 0.210

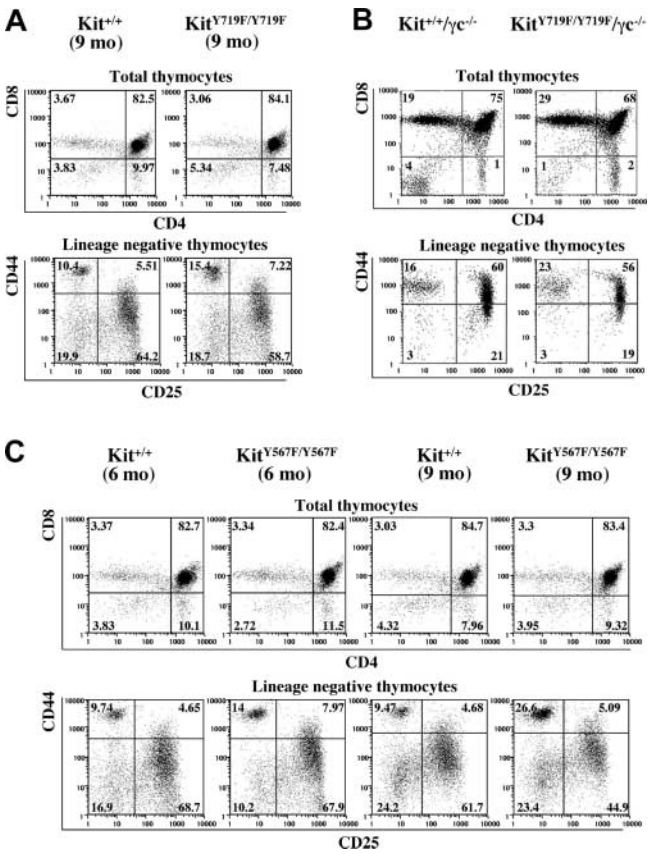

Figure 3. Age-dependent block in pro T cell development in $Kit^{Y567F/Y567F}$, but not in $Kit^{Y719F/Y719F}$ mice. (A) Expression of CD4 and CD8 was analyzed in 9-mo-old $Kit^{Y719F/Y719F}$ and littermate controls (top). Gated lineage negative thymocytes from the same animals were analyzed for CD44 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 $Kit^{+/+}$ $\gamma c^{-/-}$ and $Kit^{Y719F/Y719F}$ $\gamma c^{-/-}$ mice. (C) CD4 and CD8 expression was analyzed on $Kit^{Y567F/Y567F}$ total thymocytes and controls (top) and expression of CD44 and CD25 was analyzed in lineage-negative thymocytes (bottom) from 6- and 9-mo-old $Kit^{Y567F/Y567F}$ and control mice.

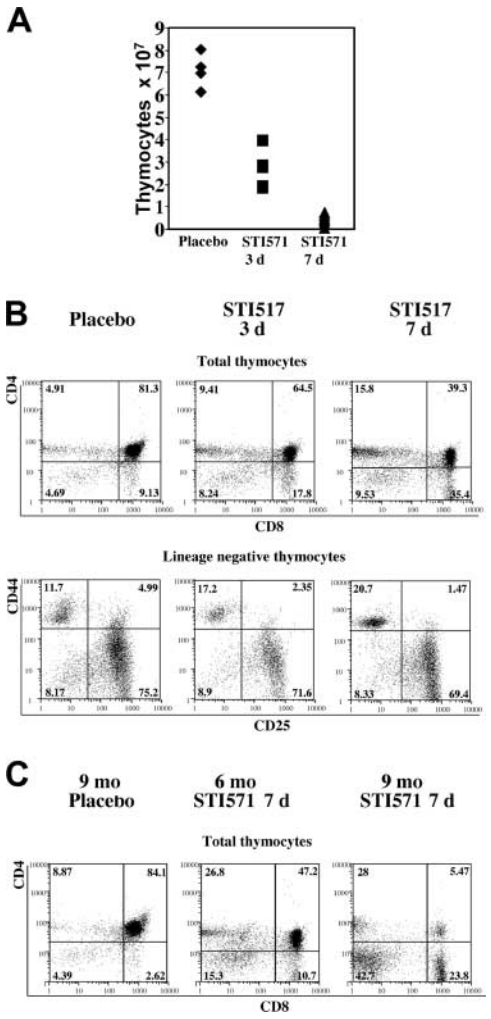

Figure 4. Age-dependent block in pro T development in C57Bl/6J mice treated with imatinib. (A) Thymus cellularity in 3–6-mo-old C57Bl/6J mice treated with imatinib 45 mg/kg twice daily for 3 or 7 d or with placebo (PBS, 7 d). The cellularity dropped from $7.15 \pm 0.8 \times 10^7$ (placebo, $n = 4$) to $2.72 \pm 0.7 \times 10^7$ (3 d treatment, $n = 6$) and $0.46 \pm 0.2 \times 10^7$ (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).

Fig. 3 C). FACS analysis of lineage negative thymocytes revealed a clear relative increase in the most immature TN1 ($CD44^+$ $CD25^-$) subset and a corresponding decrease in the downstream $CD25^+$ (TN2 and TN3) subset starting at ~6 mo and peaking at ~9 mo in $Kit^{Y567F/Y567F}$ mice. At 9 mo the increase of the TN1 subset in $Kit^{Y567F/Y567F}$ mice was threefold compared with $Kit^{+/+}$ 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 $Kit^{Y567F/Y567F}$ and Control Mice*

	Total thymocytes $\times 10^7$	Lineage negative $\times 10^6$	TN1 $\times 10^6$	TN2 $\times 10^6$	TN3 $\times 10^6$	TN4 $\times 10^6$
$Kit^{+/+}$ ($n = 5$)	6.36 ± 0.75	1.55 ± 0.17	0.17 ± 0.10	0.08 ± 0.02	1.0 ± 0.21	0.30 ± 0.08
$Kit^{Y567F/Y567F}$ ($n = 6$)	6.67 ± 1.08	0.98 ± 0.23	0.25 ± 0.08	0.04 ± 0.02	0.5 ± 0.19	0.18 ± 0.04
	$P = 0.589$	$P = 0.001$	$P = 0.137$	$P = 0.005$	$P = 0.003$	$P = 0.01$

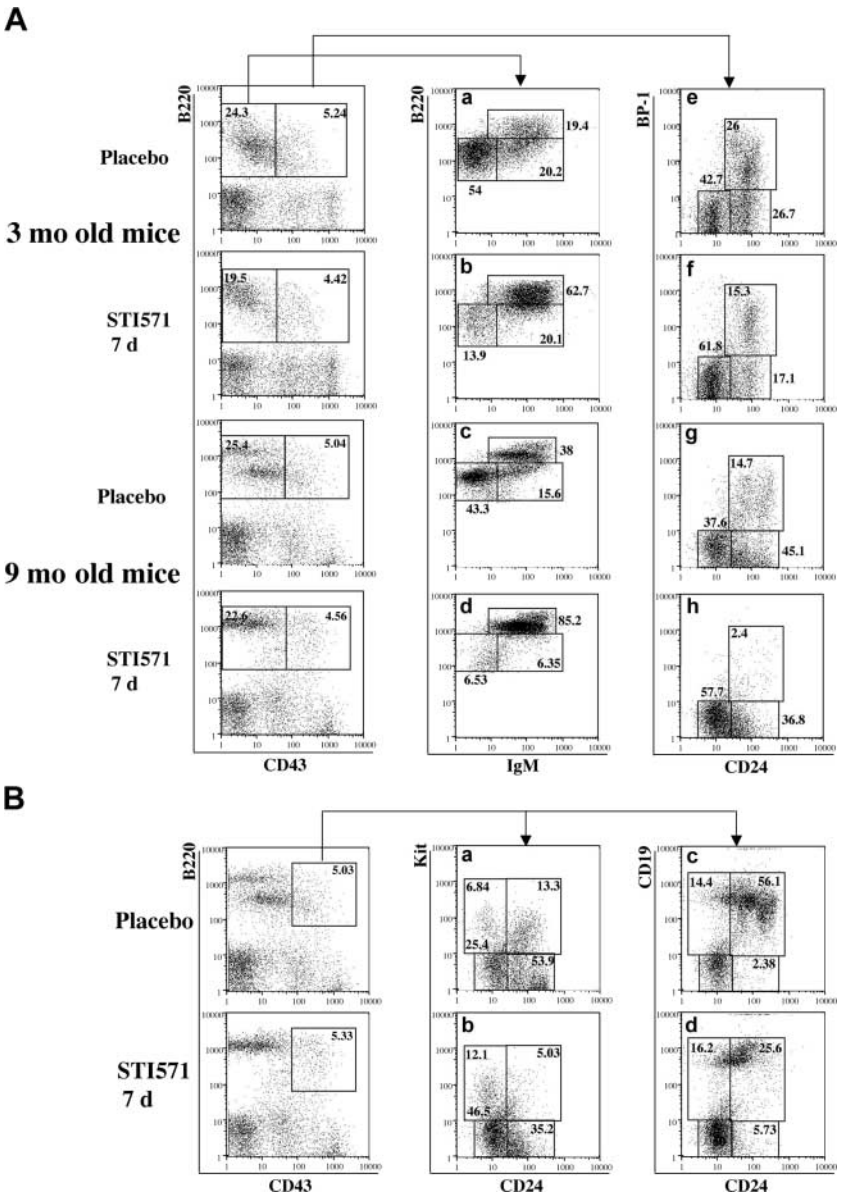

Figure 5. Age-dependent block in pro B cell development in C57Bl/6J mice treated with imatinib. (A) $B220^+$ $CD43^-$ BM cells were analyzed for IgM expression (a–d), and $B220^+$ $CD43^+$ cells were analyzed for CD24 and BP-1 expression (e–h) in 3- and 9-mo-old C57Bl/6J mice treated with imatinib 45 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, $Kit^{Y567F/Y567F}$ but not $Kit^{Y719F/Y719F}$ mice display an age-dependent effect on thymocyte development. However, this developmental retardation was incomplete and differentiation into later stages (double and single positive) did not seem to be affected. In fact, the overall cellularity of thymi in $Kit^{Y567F/Y567F}$ 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*

	Cells/femur $\times 10^7$	$B220^+/CD43^+$ $\times 10^6$	Fraction A $\times 10^6$	Fraction B $\times 10^6$	Fraction C $\times 10^6$	$B220^+/CD43^-$ $\times 10^6$	Fraction D $\times 10^6$	Fraction E $\times 10^6$	Fraction F $\times 10^6$
Placebo treated ($n = 4$)	3.05 ± 0.5	0.99 ± 0.18	0.44 ± 0.15	0.3 ± 0.15	0.24 ± 0.07	4.86 ± 1.58	2.2 ± 0.96	0.69 ± 0.21	1.82 ± 0.7
STI571 treated ($n = 5$)	2.86 ± 0.33	0.76 ± 0.09	0.41 ± 0.04	0.25 ± 0.09	0.06 ± 0.02	1.52 ± 0.34	0.07 ± 0.02	0.1 ± 0.02	1.33 ± 0.33
	P = 0.545	P = 0.07	P = 0.699	P = 0.583	P = 0.01	P = 0.02	P = 0.02	P = 0.01	0.268

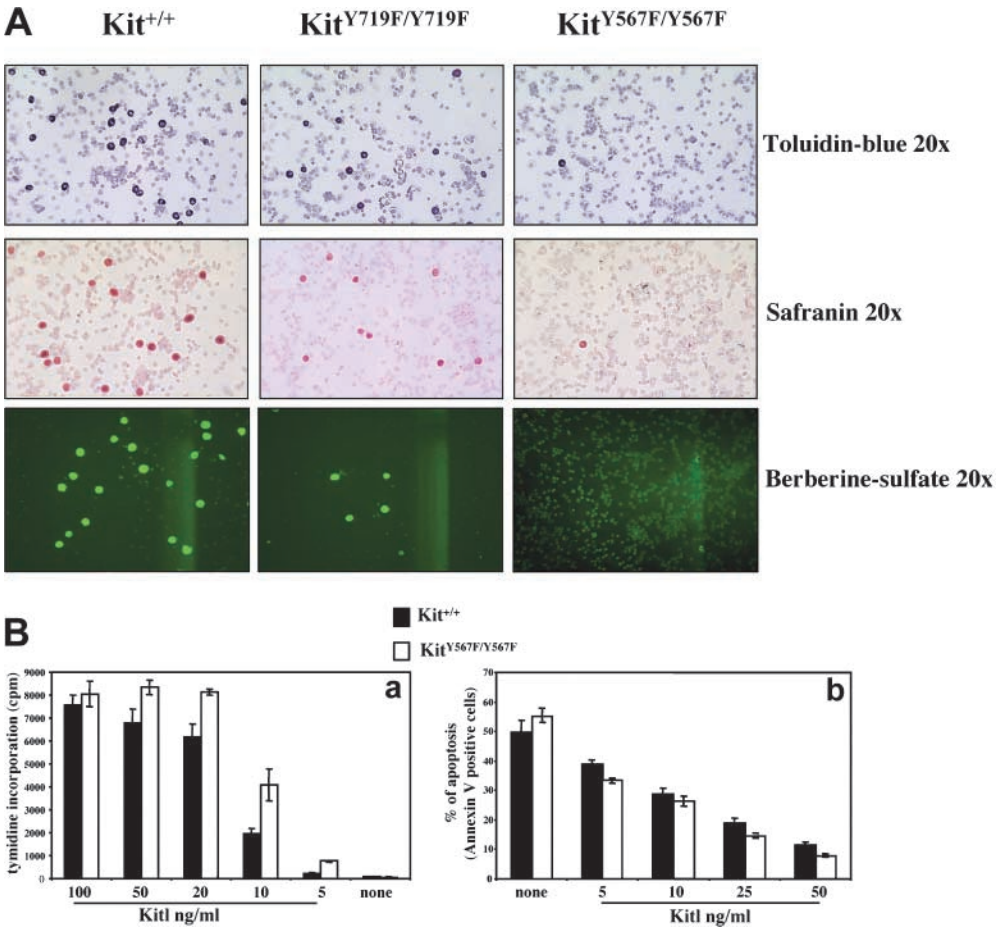

Figure 6. Analysis of mast cell characteristics in vivo and in vitro. (A) Histological analysis of peritoneal mast cells isolated from $Kit^{Y719F/Y719F}$, $Kit^{Y567F/Y567F}$, and control mice. (B) Proliferation and cell survival characteristics of $Kit^{Y567F/Y567F}$ and WT BMMC. Proliferation: Cells were stimulated with KitL (as indicated). Data are expressed as means ± standard error of triplicate samples. Apoptosis: $Kit^{Y567F/Y567F}$ and WT BMMC were incubated and in serum-free medium containing KitL as indicated for 50 h. Percentage of apoptosis was determined by annexin V staining and FACS® analysis. Experiments were done in triplicate. Similar results were obtained in three independent experiments.

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 $Kit^{W/W}$ mice. Whereas the developmental arrest is more complete in $Kit^{W/W}$ mice (9) than in $Kit^{Y567F/Y567F}$ 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® 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® 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 $CD4^+$ $CD8^+$ subset was strongly reduced, and analysis of the TN subsets revealed a partial block at the TN1 stage. The lineage negative $CD44^+$ $CD25^-$ thymocytes were increased at the expense of the $CD44^+$ $CD25^+$ and the $CD44^-$ $CD25^+$ 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 $B220^+$ $CD43^+$ $CD24^+$ $BP\text{-}1^+$ (fraction C) and $B220^+$ $CD43^-$ IgM^- (fraction D) subsets was observed similar to that seen in $Kit^{Y567F/Y567F}$ 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 $Kit^{Y567F/Y567F}$ mice, the Kit^+ and $CD19^+$ cells within the $B220^+$ $CD43^+$ $CD24^{high}$ 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, $Kit^{W/W}$ and $Kit^{Y567F/Y567F}$.

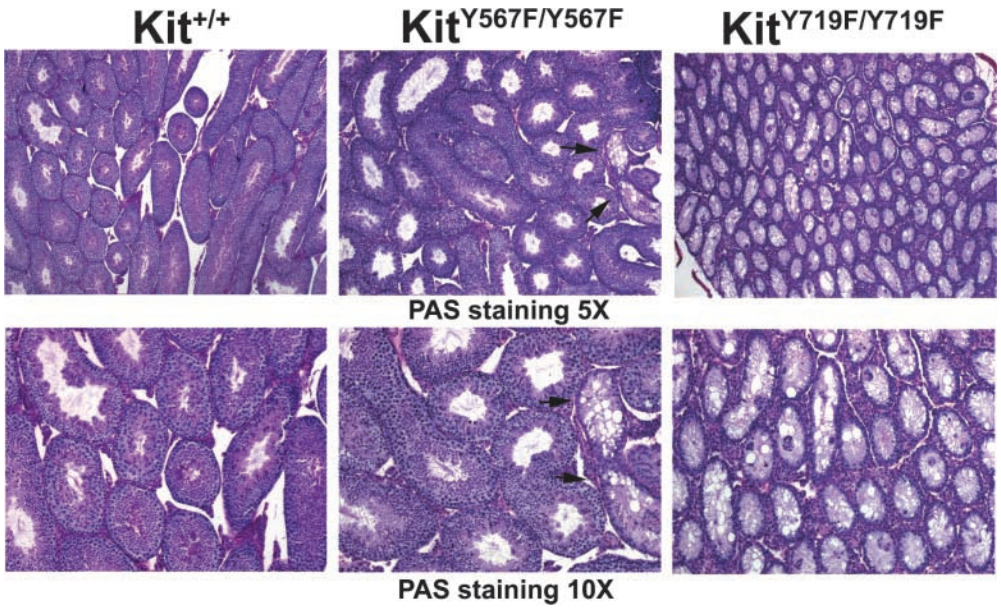

Figure 7. Histological analysis of adult testis in $Kit^{Y719F/Y719F}$ and $Kit^{Y567F/Y567F}$ mice. Paraffin sections obtained from $Kit^{+/+}$, $Kit^{Y719F/Y719F}$, and $Kit^{Y567F/Y567F}$ mice stained with hematoxylin-periodic acid-Schiff's are shown. Empty tubules in the $Kit^{Y567F/Y567F}$ testis are indicated by arrows.

Peritoneal Connective Tissue Mast Cells But Not Skin Connective Tissue Mast Cells Are Affected in Both $Kit^{Y567F/Y567F}$ and $Kit^{Y719F/Y719F}$ Mice. KitL and Kit play a critical role in mast cell development and maintenance in the adult animal (7, 8). Interestingly, both the Kit^{Y719F} and the Kit^{Y567F} 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 $Kit^{Y567F/Y567F}$ and $Kit^{Y719F/Y719F}$ mice (Fig. 6 A).

In contrast to this mast cell deficiency in vivo in $Kit^{Y567F/Y567F}$ mice, KitL-mediated proliferation of $Kit^{Y567F/Y567F}$ 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 phophatase SHP2 and the adaptor protein APS, which are affected by the Y567F mutation (15, 16). Survival was marginally enhanced in $Kit^{Y567F/Y567F}$ BMMC.

Differential Effects of the Kit^{Y567F} and the Kit^{Y719F} Mutations in Spermatogenesis. Kit^{Y567F} heterozygous and homozygous mice were viable, healthy, and they were born at Mendelian frequencies. Our previous analysis of gametogenesis in $Kit^{Y719F/Y719F}$ 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 Kit^{Y567F} 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 testis 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 Kit^{Y567F} mutation does not affect this process.

Table IV. *Number of Mast Cells in the Skin and Peritoneum of $Kit^{Y719F/Y719F}$ and $Kit^{Y567F/Y567F}$ Mice*

Genotype	Age	Skin mast cells	Peritoneal mast cells (% of total peritoneal cells)
	wk	*cells/cm*	
$Kit^{+/+}$	18–24	150 ± 9 (n = 3)	3.2 ± 0.76 (n = 3)
$Kit^{Y567F/Y567F}$	18–24	153 ± 30 (n = 3)	0.07 ± 0.056 (n = 3)
$Kit^{+/+}$ [a]	16–32	199 ± 18 (n = 3)	3.58 ± 1.69 (n = 6)
$Kit^{Y719F/Y719F}$ [a]	16–32	223 ± 13 (n = 3)	0.98 ± 0.64 (n = 5)

[a]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, Kit^{Y719F} and Kit^{Y567F}, 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-

ferent 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 α 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 Kit^{Y719F} 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 Kit^{Y719F} 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 Kit^{Y567F} mutation does not affect these processes. The Kit^{Y719F} but not the Kit^{Y567F} 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, Kit^{W}, 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 $Kit^{Y567F/Y567F}$, but not in $Kit^{Y719F/Y719F}$ 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 $Kit^{Y567F/Y567F}$ and in $Kit^{Y719F/Y719F}$ mice (unpublished data), in BM of $Kit^{Y567F/Y567F}$ 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 Kit^{Y567} 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-PY567, 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 $Kit^{Y567F/Y567F}$, but not $Kit^{Y719F/Y719F}$ 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 Kit^{Wv}, single- and double-positive thymocyte numbers in $Kit^{Y567F/Y567F}$ 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 Kit^{Y567F} mutation on thymocyte development is partial and age related. In contrast the Kit^{Y719F}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 $Kit^{Y719F/Y719F}$ and $Kit^{Y567F/Y567F}$ 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 germline Kit mutations (this paper and reference 9). The overlapping phenotypes caused by genetic and pharmacological ablation suggest that both genetic and pharmacological modes of Kit inhibition affected the same signaling pathways in vivo. Moreover, similar to mice carrying germline Kit mutations, which lack Kit function throughout live, 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 microenvironments 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).

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