FIP1L1/PDGFRα synergizes with SCF to induce systemic mastocytosis in a murine model of chronic eosinophilic leukemia/hypereosinophilic syndrome

Running title: *FIP1L1-PDGFRA*-induced systemic mastocytosis

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Supported by American Heart Association Ohio Valley Affiliate Postdoctoral Fellowship (Y.Y.), University of Cincinnati Cancer Center Grant (J.A.C), the Campaign Urging Research for Eosinophilic Disease (C.U.R.E.D, M.E.R), and the Buckeye Foundation (M.E.R.)
Abstract

Expression of the fusion gene FIP1-like 1/platelet-derived growth factor receptor alpha (FIP1L1/PDGFRα, F/P) and dysregulated c-kit tyrosine kinase activity are associated with systemic mastocytosis (SM) and chronic eosinophilic leukemia (CEL)/hypereosinophilic syndrome (HES). We analyzed SM development and pathogenesis in a murine CEL model induced by F/P in hematopoietic stem cells and progenitors (HSC/P) and T-cell overexpression of IL-5 (F/P positive CEL mice). These mice had more mast cell (MC) infiltration in the bone marrow (BM), spleen, skin and small intestine than control mice transplanted with IL-5 transgenic HSC/P. Moreover, intestinal MC infiltration induced by F/P expression was severely diminished, but not abolished, in mice injected with neutralizing anti-c-kit antibody, suggesting that endogenous stem cell factor (SCF)/c-kit interaction synergizes with F/P expression to induce SM. F/P-expressing BM HSC/P showed proliferation and MC differentiation in vitro in the absence of cytokines. SCF stimulated significantly greater migration of F/P-expressing MC than mock vector-transduced MC. F/P-expressing BMMC survived longer than mock-vector control BMMC in cytokine-deprived conditions. The increased proliferation and survival correlated with increased SCF-induced Akt activation. In summary, F/P synergistically promotes MC development, activation and survival in vivo and in vitro in response to SCF.
Introduction

Systemic mastocytosis (SM) is characterized by the accumulation of neoplastic mast cells (MC) in multiple organs and can exhibit either an indolent or an aggressive clinical course\textsuperscript{1,2}. The organs most frequently affected are bone marrow (BM), skin, liver, spleen, and the gastrointestinal tract\textsuperscript{2,3}. A subset of patients with SM has been shown to have a clonal MC and eosinophil expansion due to an interstitial deletion in chromosome 4q12, that results in the generation of a fusion gene, \textit{FIP1L1/PDGFRα} (\textit{F/P})\textsuperscript{4,5}. The \textit{F/P} fusion gene product acts as a constitutively active tyrosine kinase\textsuperscript{6}. Eosinophilia (in BM and/or peripheral blood) commonly accompanies SM (20\%–40\% of cases, termed SM-Eo or SM-CEL) and up to one half of SM-Eo patients carry the \textit{F/P} fusion gene\textsuperscript{7}. Expression of the \textit{F/P} fusion gene has been detected in MC as well as eosinophils since the \textit{F/P} mutation occurs in a multipotential haematopoietic progenitor cells capable of giving rise to multiple lineages\textsuperscript{8,9}. SM induced by constitutively active c-kit mutations and SM induced by \textit{FIP1L1/PDGFRα} appear to have distinct clinical characteristics\textsuperscript{10}. The basis for the preferential expansion of eosinophils and MC in the patients with the \textit{F/P} fusion gene remains unclear. The role of the c-kit receptor in \textit{FIP1L1/PDGFRα}-induced SM has not yet been explored.

Recently, we have established a murine \textit{F/P} positive CEL like model by retroviral introduction of the \textit{F/P} fusion gene into HSC/P in the presence of IL-5 transgene overexpression\textsuperscript{11}. The mice demonstrated severe eosinophilia with leukocytosis and tissue infiltrations of a large number of eosinophils in most organs. In these mice, like in
patients with F/P positive CEL, early hematopoietic progenitors are responsible for the F/P induction of the hypereosinophilic syndrome, implying that the F/P fusion gene may also be expressed in MC in this murine model of F/P positive CEL disease\textsuperscript{12}.

Up until now only one murine myeloproliferative SM model has been reported, based on the c-kit receptor mutation D816V that transforms c-kit to a constitutively active tyrosine kinase\textsuperscript{3,13}. We postulated that a murine model of F/P-induced SM might represent an opportunity to clarify the mechanisms of tyrosine kinase dependent SM and may be useful for developing alternative therapies in SM-CEL. In this study, we aimed to examine the impact of F/P expression on MC levels and MC distribution, activation and signaling in response to the MC growth factor c-kit ligand (stem cell factor [SCF]).
Methods

Mice
Age- and gender-matched wild type and CD2-IL-5 transgenic (Tg) BALB/c mice\textsuperscript{11,14} were used as BM donors. BALB/c (wild type) female mice (7-12 week old) were obtained from Taconic Farms (Germantown, NY). All mice were maintained under specific pathogen free conditions in Cincinnati Children’s Hospital Animal Facility. Animal protocols were approved by the Animal Care Committee of Cincinnati Children’s Hospital Medical Center.

Retroviral constructs and viral supernatants
Retroviral constructs were murine stem cell virus (MSCV)-based\textsuperscript{15} bicistronic vectors denominated MSCV-F/P-IRES-EGFP and MSCV-IRES-EGFP (mock vector) (kindly provided by Drs. Gary Gilliland and Jan Cools, Harvard Medical School, U.S.A.). Efficient expression of F/P by this retroviral vector has been previously shown\textsuperscript{16,17}. Retrovirus supernatant was generated in the Phoenix-gp cells\textsuperscript{17} as previously described\textsuperscript{18}.

Retroviral transduction into hematopoietic progenitor cells
Mice were treated with 150 mg/kg of 5-fluorouracil (5-FU) administered intraperitoneally beginning 6 days prior to BM harvest. Femora, tibiae and iliac crests were harvested and their BM content was isolated. Low-density BM (LDBM) cells were separated by density gradient fractionation according to manufacturer’s instructions (Histopaque 1083, Sigma-Aldrich, St. Louis, MO), pre-stimulated in the presence of recombinant mouse (rm) IL-3 (6 ng/ml, PeproTech, Rocky Hill, NJ), recombinant rat stem cell factor (rrSCF, 10 ng/ml,
Amgen, Thousand Oaks, CA), and rmIL-6 (10 ng/ml, PeproTech, Rocky Hill, N.J.) and transduced during two days in a protocol that included two rounds of spinoculation (1,800 x g for 90 min) with retroviral supernatants separated by 24 hours of incubation, and followed by 6 additional hours of incubation at 37°C, 5% CO₂. Transduction efficiency of all experiments was ~10% without any significant difference among the different groups as previously described11.

**Hematopoietic stem/progenitor cell (HSC/P) transplantation**

Retrovirally transduced cells were transplanted 6 hours after the second round of spinoculation/transduction. A total of 2.5-6.5 x 10⁶ cells/mouse were injected into the lateral tail vein of previously lethally irradiated (4.5Gy x 2 doses, 3 hours apart, ¹³⁷Cs source, dose rate 60-65 cGy/min) recipient mice (BALB/c, wild type)11.

**Antibody administration**

To deplete MC, mice were administered five doses (first dose intravenously, then intraperitoneally) of 1 mg of anti–c-kit Ab (ACK2) or a control Ab (J1.2) starting 3 weeks after transplantation as previously described19. All mice were sacrificed 2 days after the last injection.

**Histopathology**

For tissue histology, relevant organs were fixed in 10% buffered formalin and embedded in paraffin. The tissue sections were stained for chloroacetate esterase (CAE) activity and lightly counterstained with methyl-green as previously described19,20. At least three
random sections per mouse were analyzed. Quantification of stained cells per square millimeter of intestinal lamina propria and epidermis and dermis was performed by blind morphometric analysis using ImagePro Plus version 4.1 (Media Cybernetics, Silver Spring, MD)\textsuperscript{11,19}. Masson trichrome staining was performed as previously described\textsuperscript{21} where blue deposits denote abundant collagen deposition.

**MC content analysis by flow cytometry**

BM and spleen cell suspensions were obtained after mechanical treatment as previously described\textsuperscript{22}. Intestinal cell suspensions were obtained from the ileum. After washing in phosphate buffered saline (PBS), the tissue was minced, treated with collagenase (0.12 mg/mL, Liberase CL, Roche, Indianapolis, IN) and DNAse (0.25mg/ml, Sigma, St. Louis, MO) for 30 minutes at 37° C and then squeezed through 40 µm-mesh filters. Cells were resuspended in RPMI-1640 with 10% fetal calf serum (FCS)\textsuperscript{23}. Single-cell suspensions were stained with the following mAbs: PECy7-conjugated anti-CD45 (clone 30F-11, BD-Pharmingen, San Jose, CA), APC-conjugated anti-ckit (clone 2B8, BD-Pharmingen), PE-conjugated anti-FcεRIα (clone MAR-1, eBiosciences, San Diego, CA). Dead cells were excluded from analysis by staining with 7-aminoactinomycin D (7-AAD) (Molecular Probes, Eugene, OR). Multicolor flow cytometric analysis was performed with a FACSscalibur or a FACSCanTo flow cytometer, and the data were analyzed using CellQuest or FACSDiVa softwares (Becton Dickinson, San Jose, CA).

**Plasma mouse MC protease-1 (MMCP-1) concentration analysis**

The plasma level of MMCP-1 was measured by ELISA according to the manufacturer’s
instructions (Mouse MMCP-1 OptEIA ELISA set, BD Biosciences Pharmingen)\textsuperscript{19}.

**In vitro MC development**

Retrovirally-transduced EGFP\textsuperscript{+}HSC/P (empty vector and F/P-expressing) were sorted (FACS Vantage SE DiVa) two days after the last transduction round (day 0 of culture). Sorted cells were cultured at a starting density of 1-2 \times 10^5 cells/ml in IMDM supplemented with L-glutamine, penicillin, streptomycin, 10% FCS (Invitrogen), and either no cytokines or supplemental cytokines (100 ng/mL rrSCF and/or 100 ng/mL of rmIL-3 (Peprotech)). The cytokines were added every 2-3 days and the medium was renewed every week. Migration and survival experiments were performed in cultures containing > 90% MC after a minimum of 4 weeks of culture\textsuperscript{24,25}.

**Bone marrow-derived MC (BMMC) chemotaxis assay**

Migration assays in response to SCF-induced chemotactic gradient were performed by seeding 500,000 BMMC on 8-µm pore size, polycarbonate membrane transwells (Corning Inc, Lowell, MA), in triplicate, on a gradient formed by 0, 1, 10 or 100 ng/mL SCF for 4 hours at 37°C, 5% CO\textsubscript{2}\textsuperscript{25}. The migrated cells in the bottom chamber were collected, resuspended and divided in aliquots for cell enumeration.

**Survival assay of BMMC**

BMMC were starved in IMDM supplemented with 10% FCS in absence of cytokines for 48 hours and stained with Annexin-V-APC (BD-Pharmingen) and 7-AAD according to manufacturer’s instructions. Survival was analyzed as the percentage of Annexin-V\textsuperscript{-}/7-
AAD− events\textsuperscript{25}.

**Immunoblotting**

After starvation for 24 hours in IMDM containing 1% BSA, BMMC were lysed in a buffer containing 10 mM TrisHCl pH 7.4, 130 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and protease and phosphatase inhibitors (5 mM EDTA, 5 mM EGTA, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na\textsubscript{3}VO\textsubscript{4}) and Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). The lysates were incubated on ice for 20 min, cleared by centrifugation at 12,000 x g for 15 min, and separated on a 10% SDS-PAGE gel. For western blot analysis, proteins were transferred onto a PVDF membrane, blocked with 5% BSA in TBS-0.1% Tween 20, and probed with antibodies specific for phospho-Erk (p42/p44 MAPK, Thr202/Tyr204; 1:1000 dilution), phospho-Akt (Ser473; 1:1000), phospho-STAT5 (Tyr694; 1:1000), phospho-c-kit (Tyr719; 1:1000); all from Cell Signaling Technology (Danvers, MA), phospho-Tyr (4G10, 1:1000; Upstate, Billirica, MA), or β-actin (AC-15; 1:5000; Sigma). Blots were then incubated with HRP-conjugated secondary antibodies (Cell Signaling Technology), and developed with LumiGLO reagents (Cell Signaling Technology) \textsuperscript{25}.

**Statistical analysis**

Data were expressed as mean ± standard error of the mean except when otherwise stated. Statistical analysis of data was performed by Student \textit{t} test when comparing 2 groups and one-way or two-way analysis of variance (ANOVA) followed by multiple comparisons.
for 3 or more groups. When non-parametric analyses were required, statistical
comparisons were performed with either Mann-Whitney $U$ test for 2 groups and Kruskal-Wallis test for 3 or more groups. $P$ values < 0.05 were considered significant.
Results

Multi-organ infiltration of F/P-expressing MC in F/P positive CEL mice

We initially investigated whether F/P positive CEL mice demonstrate tissue MC infiltration. The MC content of organs from mice with overt CEL\textsuperscript{11} was analyzed at 4-5 weeks post-transplantation by CAE staining in tissue sections and flow cytometry of cell suspensions. The F/P positive CEL mice showed ~10-fold higher levels of MC infiltration in the small intestine compared to mice transplanted with donor-matched, mock vector-transduced HSC/P (Figure 1A and B). Interestingly, the intestinal MC infiltration of F/P positive CEL mice was primarily associated with MC residing in the lamina propria and intraepithelial locations associated with villi, whereas MC in control mice were primarily in the crypt areas, similar to what has been described in human intestinal mastocytosis\textsuperscript{20,36}. This data suggested that MC in F/P positive CEL mice might possess higher migration activity to epithelium in vivo.

The skin is one of the most common organ involved in patients with mastocytosis and HES. Cutaneous MC were detected in the dermis in both F/P positive CEL mice and control mice; however, the cutaneous MC level was significantly increased in F/P positive CEL mice compared to vector control mice (Figure 1C). To determine whether the MC infiltration was affecting multiple organs, we analyzed the content of F/P-expressing cells based on the expression of EGFP. The content of EGFP\textsuperscript+ and EGFP\textsuperscript- MC (c-kit\textsuperscript+/Fc\varepsilon RI\alpha\textsuperscript+ cells) in single-cell suspensions of BM and spleen (Figure 2A-B) and
small intestine (CD45+/c-kit+/ FcεRIα+ cells; **Figure 2C**) was analyzed by flow cytometry. The vast majority of MC infiltrating BM, spleen and intestine of F/P positive CEL mice expressed EGFP, while there was no expansion of the EGFP-negative MC population compared to the mock-vector control, demonstrating that SM induced in these mice was due to expression of F/P.

BM sections of F/P positive CEL mice demonstrated the presence of increased collagen deposits compared with control mice (Supplementary Figure 1), a finding frequently associated with myeloproliferative disorders\(^26\). MC in F/P positive CEL mice were dysmorphic, with irregular shape and frequent cytoplasm extensions (examples of intestinal MC are shown in **supplementary Figure 2A-B**), compared with the relatively round shape of MC in control mice. This irregular shape is reminiscent of “spindle shape” found in clinical systemic mastocytosis.

To determine whether IL-5 overexpression had any significant role in the induction of mastocytosis in these mice, we analyzed MC tissue infiltration in mice transplanted with non-IL5Tg, mock-transduced and non-IL5Tg, F/P-expressing BM cells. IL-5 overexpression did not significantly modify the MC tissue content in mice engrafted with mock-transduced HSC/P. However, the intestinal MC infiltration in mice transplanted with F/P-transduced HSC/P was ~2-fold higher when IL-5 was overexpressed (**Supplementary Figure 3A**). IL-5 overexpression did not significantly change the MC
infiltration of the skin of mice receiving either mock- or F/P-transduced HSC/P transplants (Supplementary Figure 3B).

Similar to serum tryptase determination in SM patients, a systemic assay of MC content and degranulation in the mouse is the determination of circulating mouse MC protease (MMCP)-1 levels. The levels of plasma MMCP-1 were extremely elevated in F/P positive CEL mice (averaging 1,684-fold higher than in control mice, Figure 3), indicating the presence of a large MC burden in these mice.

Similar to the observation of intestinal MC infiltration, IL-5 overexpression did not significantly modify the MMCP-1 serum levels of mice receiving mock-transduced HSC/P transplants but it increased the MMCP-1 serum levels of mice transplanted with F/P-transduced HSC/P by ~ 2-fold (Supplementary Figure 3C).

SCF synergizes with F/P expression to induce MC infiltration in F/P positive CEL mice

Since c-kit receptor-dependent signaling is crucial for normal MC development and function, we analyzed whether F/P-induced SM was still c-kit dependent. To reduce or eliminate c-kit signaling, we used parenteral administration of an anti-c-kit blocking antibody (ACK.2) that specifically neutralizes the c-kit receptor and has been shown to completely abolish MC infiltration in models of allergen-induced mastocytosis. Since
ACK2 antibody may interfere with HSC/P function when administered during the engraftment process\textsuperscript{28}, we administered this antibody starting on day +21 post-transplantation when the engraftment of HSC/P is considered largely complete. To verify this point, we analyzed the white blood cell count of mice two days after the administration of the last dose of antibodies. Administration of ACK2 antibody did not significantly change the total white blood cell count of mice compared with an isotype control antibody after transplantation of IL-5Tg, F/P-expressing HSC/P (8.8 ± 4.4 and 5.5 ± 1.4 x 10\textsuperscript{3}/mm\textsuperscript{3}, respectively, p=N.S.).

Anti-c-kit antibody administration significantly diminished SM (Figures 4A and 4B), suggesting that c-kit-mediated signaling is partly responsible for the SM of F/P positive CEL mice. Likewise, flow cytometry analysis of single-cell suspensions of BM, spleen and small intestine showed that the administration of anti-c-kit antibody significantly decreased EGFP\textsuperscript{+} MC infiltration in spleen and small intestine (Table 1) although not in the BM. The systemic burden of MC (as determined by elevation of MMCP-1) was diminished by the anti-c-kit antibody treatment (Figure 4C). These data suggest that tissue infiltrations of F/P\textsuperscript{+} MC are associated with SCF/c-kit signaling.

**F/P fusion protein induces increased survival of BMMC and specifically synergizes with SCF to induce ex vivo MC output**

To determine the role of F/P on preventing BMMC apoptosis, their survival after cytokine deprivation was analyzed by flow cytometry in an Annexin-V binding assay.
Expression of the F/P fusion gene was associated with significantly reduced apoptosis of BMMC in cytokine-deprived conditions \((\text{Figure 5A})\). To examine if F/P expression favors BMMC expansion in culture, F/P fusion- or empty vector-transduced wild type BM-HSC/P were cultured in the absence or presence of SCF and/or IL-3 (followed for up to 4 weeks, \(\text{Figure 5B-D}\)). In these conditions, F/P-expressing BM HSC/P expanded modestly into a differentiated MC population \((\text{Figure 5B})\). Expansion of F/P-expressing MC was significantly higher in the presence of SCF \((\text{Figure 5C}, \sim 70\text{-fold higher than the culture without cytokines on week 4 of culture})\). In contrast, mock vector-transduced BM-derived HSC/P in the absence of cytokines were not able to differentiate into MC and SCF alone induced negligible MC output from mock vector-transduced BM-HSC/P \((\text{Figure 5B and C})\). While IL-3 induced a striking expansion of MC from mock vector-transduced HSC/P as previously shown by others\(^{24,25}\), it had only a marginal effect on MC output from F/P\(^+\) HSC/P beyond the level observed in non-cytokine containing cultures \((\text{Figure 5D})\). Taken together, these data show the expansion of MC output is enhanced by F/P in a synergistic fashion with SCF but not with IL-3.

**F/P expression synergizes with SCF to induce Akt activation**

In order to evaluate the signaling pathways activated by F/P and the observed synergism in MC growth, we examined downstream signaling pathways implicated in SCF/c-kit ligand/receptor interactions in MC. Since Akt is a known downstream effector of c-kit receptor signaling and has also been implicated in the survival, proliferation and migration of several cell types\(^{29}\) including F/P transduced human CD34\(^+\) cells\(^{30}\), we
analyzed Akt activation after SCF stimulation. As seen in Figure 6A (upper panel) and Supplementary Figure 4, SCF induced a ~6.5-fold increased Akt activation in F/P-expressing BMMC compared with mock vector-transduced cells. This activation was prevented by preincubation with AktVIII, an Akt activation inhibitor, which did not modify SCF-dependent Erk or STAT5 activation levels (Supplementary Figure 5). Interestingly, there were neither significant differences in the level of c-kit activation after SCF stimulation between mock-transduced and F/P-expressing MC or in the levels of phosphorylated F/P when F/P-expressing MC were stimulated with SCF (Figure 6A, second and third panels). At the same time, there was no synergistic effect of c-kit activation and F/P on STAT5 or Erk activation (Figure 6A, fourth and fifth panels), suggesting a specific synergistic stimulation of Akt signaling pathway by F/P and SCF, downstream both c-kit and F/P.

**F/P⁺ BMMC show enhanced migration in response to SCF**

Finally, because we observed increased numbers of MC infiltrating different tissues and a synergism with SCF in the expansion of BMMC and Akt activation and since SCF is a well-described chemotactic factor for MC, we next determined if the expression of F/P affected the motility of MC in response to SCF. BMMC expressing either mock vector or F/P were generated in cultures containing both IL-3 and SCF and tested in an SCF-dependent transwell migration assay. MC migration through a SCF gradient was significantly higher (~3-fold increase for 10 and 100 ng/mL SCF gradients) for F/P-
expressing BMMC than those of mock vector (Figure 6B). These data are consistent with the systemic tissue infiltration of MC found in vivo in F/P positive CEL mice.
Discussion

F/P-induced disease is characterized by increased cell numbers and activation of the MC and eosinophil lineages. F/P expression leads to clinical SM and CEL (SM-CEL) with infiltration of hematopoietic tissues and non-hematopoietic tissues. Among the non-hematopoietic tissues, the skin and intestine are commonly involved.

There has been a significant degree of controversy on the role of FIP1L1/PDGFRα in SM. Mutation of the PDGFRA gene has been found in eosinophils and MC in SM-F/P positive CEL or gastrointestinal stromal tumors, which do not have KIT mutations pointing out the involvement of common downstream signaling pathways common to c-kit and PDGFRA in the development of different diseases. This suggests the potential influence of cell type-specific activation pathways and the possible role of stromal expression of SCF in the pathogenesis of SM. This controversy has been due, at least partly, to the absence of satisfactory pathogenetic models for SM-F/P positive CEL, which would allow the study of the intrinsic role of F/P-expressing MC in vivo and in vitro.

Here we show that a murine model of F/P positive CEL also demonstrates biological features comparable with those of SM. This model of SM is a direct result of the expression of F/P in hematopoietic progenitors and MC since despite hypereosinophilia, the vast majority of tissue-infiltrating MC express F/P while there is no significant expansion of F/P-negative MC. This model of SM-F/P positive CEL demonstrates increased levels of F/P-expressing MC in the skin, small intestine, BM and spleen. This model, similar to some
models of parasite-induced mastocytosis, demonstrates MC infiltration within the intestinal epithelium of F/P positive CEL mice\textsuperscript{20,36}. SCF-expressing mesenchymal cells surrounding the intestinal epithelium are thought to nurture the intestinal stem cell niche composed of intestinal stem cells and progenitors\textsuperscript{37}. These mesenchymal cells generate a gradient of SCF that may attract large numbers of proliferating MC in SM, leading to their presence in the intestinal epithelium. An increased migration response to SCF by F/P-expressing MC would explain their preferential localization in the epithelial layer within the intestine.

We hypothesized that preferential MC development associated with F/P fusion in vitro and in vivo would be dependent on synergistic effects of SCF with the F/P fusion protein. Indeed, MC intestinal and spleen infiltrations and the elevated levels of MMCP-1 induced by F/P expression were significantly diminished but not abolished by administration of an anti-c-kit antibody, suggesting that SCF/c-kit interaction acts synergistically with F/P expression to induce mastocytosis. Interestingly, BM MC infiltration was not decreased after administration of anti-c-kit antibody. Since ACK2 appears to adequately target c-kit expressing BM cells\textsuperscript{28}, these data suggest that BM MC are less dependent on SCF signaling to survive in vivo. While F/P\textsuperscript{+} BMMC differentiation into MC in vitro occurred even in the absence of cytokines, such expansion was augmented in the presence of SCF alone. Although SCF is a key cytokine in the development, activation and survival of MC, SCF alone is not sufficient for the development of MC from BM cells as this process requires co-factors such as IL-3 and IL-4\textsuperscript{38,39}. Interestingly, SCF does not require any MC-extrinsic cofactors to develop F/P\textsuperscript{+} MC from F/P\textsuperscript{+} HSC/P, suggesting that F/P fusion may alternatively work as a co-factor.
Interestingly, F/P not only induces an increased response to SCF, but it also synergizes with SCF to induce increased MC proliferation and survival. We found cooperation between SCF and F/P-dependent signaling to specifically activate Akt which correlated with increased proliferation and migration. However, there was not a consistent activation of Erk or STAT5 by F/P or cooperation with SCF in hyperactivation of Erk or STAT5, suggesting that Erk and STAT5 activation do not have major roles in F/P-induced mastocytosis. Interestingly, the absence of an effect by IL-3 on proliferation of F/P-expressing MC, suggests that IL-3 does not modify the level of tyrosine kinase dependency of the MC in this murine model. Thus, SM induced by F/P in this murine model could be “tyrosine kinase addicted”. These findings support the importance of SCF in F/P-associated MC development, survival and activation, and the cooperative signaling of multiple tyrosine kinases in neoplasia.

In summary, our results show that the F/P fusion gene induces mastocytosis characterized by increased levels of MC in hematopoietic organs, skin and small intestine, and elevated serum levels of MMCP-1, along with prolonged survival and higher migration activity of MC in response to SCF. It is likely that these F/P MC directly contribute to the disease pathogenesis, as patients with this subset of disease have increased tissue damage including fibrosis. The finding that two tyrosine kinases (PDGFRA and c-kit) cooperate in the pathogenesis of SM-F/P positive CEL, highlights the potential utility of blocking single or multiple kinases in these patients.
Contribution: Y.Y., A. S-A., E.B.B., M.M., N. J. A. and J.A.C. performed experiments; F.D.F. analyzed results and provided anti-c-kit and isotype control antibodies for in vivo neutralization; Y.Y., D.A.W, J.A.C. and M.E.R. designed the research, interpreted the results and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


Table 1. Frequency of tissue EGFP⁺ MC found after treatment with anti-c-kit antibody

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<th>Mock-vector Anti-c-kit</th>
<th>F/P-CEL Isotype control</th>
<th>F/P-CEL Anti-c-kit</th>
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Data are shown as mean ± standard deviation, *p <0.05 compared to isotype matched control-treated mice in each group.
FIGURE LEGENDS:

Figure 1. F/P positive CEL mice have MC infiltration in relevant non-hematopoietic organs. (A) Levels of mast cells in the small intestine. Levels of intestinal mast cells in F/P positive CEL and CD2-IL-5Tg/mock vector-transduced recipient mice were assessed after development of disease (at 4-5 weeks post transplantation) by morphometric analysis of chloroacetate esterase (CAE) stained cells. Results are shown as mean ± SEM from 4 mice per group of one representative experiment (n = 3 experiments). *P< 0.001). (B) Representative small intestine section from F/P-introduced F/P positive CEL and control mice with CAE staining (original magnification, ×125), indicating a preferential intraepithelial localization of MC in F/P positive CEL mice. (C) Levels of cutaneous mast cells in F/P-induced F/P positive CEL and donor-matched control mice were assessed as in Fig. 1A. Results are shown as mean ± SEM from 4 mice per group of one representative experiment. Three independent experiments were conducted with similar results; *p< 0.05.

Figure 2. MC intrinsic expression of F/P induces SM. Frequency of viable MC was analyzed by flow cytometry of ckit⁺/FcεRIα⁺ cells on gated EGFP⁺ or EGFP-negative cells of BM (A) and spleen (B), and of viable CD45⁺/kit⁺/FcεRIα⁺ cells on gated EGFP⁺ or EGFP- cells of small intestine (C). *p<0.05 compared to EGFP-negative MC content. # p<0.05 compared to mock vector transduced (EGFP⁺) MC content. Results are shown as mean ± SEM from 4 mice per group pooled from two independent experiments.
Figure 3. Mice with F/P-induced SM have elevated serum levels of MMCP-1. Levels of MMCP-1 in CEL mice and mock vector-transduced recipient mice were assessed after development of disease (at 4-5 weeks post transplantation) by ELISA. Each dot represents results from one mouse and the horizontal bars show mean values (n=6-8, *p<0.0001). The results are pooled from two independent experiments.

Figure 4. Anti-c-kit treatment dramatically reduces the tissue MC infiltration and serum level of MMCP-1. After hematopoietic engraftment, on day +21 post-transplantation, mice were treated with an anti-c-kit neutralizing antibody (ACK2) or an isotype matched control antibody (J1.2). The effect of ACK2 treatment in the levels of intestinal (A) and skin (B) mast cells in F/P positive CEL and mock vector-transduced recipient mice were assessed by morphometric analysis of chloroacetate esterase (CAE) stained cells. Results are shown as mean ± SEM from 4-6 mice per group of one representative experiment out of two independent experiments. *p<0.05, # p<0.0001, compared to isotype control-treated mice. (C) The effect of ACK2 treatment on levels of MMCP-1 in the same groups of mice was assessed by ELISA. Each dot represents results from each individual mouse and horizontal bars show mean values (n=7-11 pooled from two independent experiments, **p<0.001).
Figure 5. F/P fusion promotes differentiation and proliferation of BMMC and increases survival after cytokine depletion. (A) F/P-expressing or mock vector-transduced BMMC were cultured in the absence of cytokines for 48 hours and then the survival of BMMC were assessed by annexin-V and 7AAD using flow cytometry. Data represents mean ± SEM of BMMC that were annexin-V and 7AAD double negative. * p<0.05 (n=3, from one representative experiment out of 3). (B-D) BMMC output associated with FIP1L1/PDGFRα expression. F/P- (squares) or mock vector (triangles) transduced 5-FU-treated BM HSC/P cells were cultured in the absence (B) or presence of recombinant stem cell factor (rSCF) (100 ng/ml, C) or rIL-3 (100 ng/ml, D). MC content of cell cultures from B-D was assessed by flow cytometry of EGFP+/ckit+/FcεRIα+ cells. The data was depicted as fold expansion (MC [EGFP+/ckit+/FcεRIα+ cell] numbers divided by seeded HSC/P numbers in indicated periods). *p < 0.05, **p<0.01 and ***p<0.001 compared to mock vector (EGFP+) MC cells (n=3 from one representative experiment out of 2 or 3 experiments).

Figure 6. Role of SCF in migration and signaling activation in F/P-expressing BMMC. (A) Time course of SCF-induced activation of Akt (p-Akt), Erk (p-Erk), STAT5 (p-STAT5), c-kit (p-c-Kit) and overall tyrosine phosphorylation (p-Tyr) in mock vector-transduced and F/P-expressing MC. β-actin is used as loading control. The values represent the densitometric quantification of p-AKT relative to β-actin in this experiment. One representative experiment (out of 3-4 completely independent experiments) is shown. (B) Migration of F/P-expressing BMMC toward stem cell factor
(SCF). F/P-expressing (solid bars) or mock vector-transduced (empty bars) BMMC were allowed to migrate in response to a gradient of different concentrations of SCF. Data represent mean ± SEM of BMMC that migrated into lower chambers. *p<0.05, compared to mock vector-transduced in each concentration of SCF; # p<0.05, compared to medium control in each group (n=3, from one representative experiment, out of 3 independent experiments with similar results).
Figure 1

(A) Bar graph showing the number of mast cells/mm². The x-axis represents the groups, Mock-vector and F/P-CEL, and the y-axis represents the mast cell count. There is a significant difference indicated by an asterisk (*).

(B) Two images showing histological sections. The left image represents Mock-vector, and the right image represents F/P-CEL. The images show differences in mast cell distribution.

(C) Bar graph with a similar format to (A), comparing mast cell counts for Mock-vector and F/P-CEL. The asterisk (*) indicates statistical significance.
Figure 2

A

Mock-vector  F/P-CEL

\[
\begin{align*}
\% c_k i_t^+ / F_c e R\alpha^+ \\
\end{align*}
\]

BM

B

Spleen

C

Small intestine
Figure 3

Mock-vector

F/P-CEL

MMCP-1 (pg/ml)

*
Figure 4

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mast cells/mm²</th>
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<tbody>
<tr>
<td>Mock-vector</td>
<td>-</td>
</tr>
<tr>
<td>Mock-vector</td>
<td>-</td>
</tr>
<tr>
<td>F/P-CEL</td>
<td>700 ± 20</td>
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<td>F/P-CEL</td>
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Anti-ckit Ab: -
Isotype control Ab: +

B

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<td>Mock-vector</td>
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<td>Mock-vector</td>
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<td>F/P-CEL</td>
<td>150 ± 15</td>
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<tr>
<td>F/P-CEL</td>
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Anti-ckit Ab: -
Isotype control Ab: +

C

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<th>Treatment</th>
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<tr>
<td>F/P-CEL</td>
<td>100000 ± 10000</td>
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Anti-ckit Ab: -
Isotypic control Ab: +
Figure 5

A

Viability (Annexin-V/7-AAD⁻, %)

Mock

F/P

B

MC output (cell fold-expansion)

Weeks

C

MC output (cell fold-expansion)

Weeks

D

MC output (cell fold-expansion)

Weeks
Figure 6

A

<table>
<thead>
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<th>SCF (min):</th>
<th>control BMMC</th>
<th>F/P+ BMMC</th>
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<tbody>
<tr>
<td>0 5 15 30</td>
<td>0 5 15 30</td>
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<tr>
<td>p-Akt (Ser473)</td>
<td>0.0 1.0 1.7 0.9</td>
<td>0.0 4.1 3.9 1.9</td>
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<tr>
<td>p-c-Kit (Tyr719)</td>
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<tr>
<td>p-Tyr</td>
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<tr>
<td>p-Erk (Thr202/Tyr204)</td>
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<td></td>
</tr>
<tr>
<td>p-STAT5 (Tyr694)</td>
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</tr>
<tr>
<td>β-actin</td>
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<td></td>
</tr>
</tbody>
</table>

B

Migration (cells) vs. SCF (ng/ml)

- * indicates significant difference from control.
- # indicates significant difference from F/P+ BMMC.

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FIP1L1/PDGFRα synergizes with SCF to induce systemic mastocytosis in a murine model of chronic eosinophilic leukemia/hypereosinophilic syndrome

Yoshiyuki Yamada, Abel Sanchez-Aguilera, Eric B Brandt, Melissa McBride, Nabeel JH Al-Moamen, Fred D Finkelman, David A Williams, Jose A Cancelas and Marc E Rothenberg