Title

Blood CD34-c-Kit+ cell rate correlates with aggressive forms of systemic mastocytosis and behaves like a mast cell precursor

Brief running head: Identification of a new blood mast cell precursor

Authors:

Sophie Georgin-Lavialle1,2*, Ludovic Lhermitte3*, Cédric Baude4*, Stéphane Barete2,4,5, Julie Bruneau2, Jean-Marie Launay6, Marie-Olivia Chandesris1, Katia Hanssens7, Christian de Gennes8, Gandhi Damaj9, Fanny Lanternier10, Mohamed Hamidou11, Olivier Lortholary4,10, Patrice Dubreuil4,7, Frédéric Feger2, Yves Lepelletier2**, Olivier Hermine1,2,4**.

Affiliations:

1 Service d’Hématologie Adultes et centre de référence sur les mastocytoses, Hôpital Necker-Enfants malades ; Université Paris Descartes, Assistance Publique-Hôpitaux de Paris, 161 Rue des Sèvres, 75743 Paris Cedex 15, France.

2 CNRS UMR 8147, Hôpital Necker-Enfants malades ; Université Paris Descartes, 161 Rue des Sèvres, 75743 Paris Cedex 15, France.

3 Laboratoire de cyto-hématologie, Hôpital Necker; Université Paris Descartes, Assistance Publique-Hôpitaux de Paris, 161 Rue des Sèvres, 75743 Paris Cedex 15, France.

4 AFIRMM (Association Française pour les Initiatives de Recherche sur le Mastocyte et les Mastocytoses), 3 Avenue George V, 75008 Paris, France.


6 Laboratoire de biochimie et biologie moléculaire, INSERM U942, Hôpital Lariboisière, Assistance Publique-Hôpitaux de Paris.
INSERM UMR 891, Centre de Recherche en Cancérologie de Marseille, Laboratoire d'Hématopoïèse Moléculaire et Fonctionnelle, Marseille, France.

Service de médecine interne 2, Hôpital Pitié-Salpêtrière, 47 Bd de l’hôpital 75013 Paris, France.

Service des Maladies du Sang, Centre Hospitalier Universitaire, Hôpital Sud, Avenue Laennec, 80054 Amiens, France

Service des Maladies infectieuses et tropicales, et centre de référence sur les mastocytoses, Hôpital Necker-Enfants malades, Université Paris V.


* Both authors contributed equally to this work
**Both authors co-directed this work.

**Corresponding author:** Pr. Olivier Hermine
Service d’Hématologie Adultes, CNRS UMR 8147 et centre de référence sur les mastocytoses,
Hôpital Necker-Enfants malades
Université Paris Descartes
Assistance Publique-Hôpitaux de Paris
149 Rue des Sèvres, 75743 Paris Cedex 15, France.
E-mail: ohermine@gmail.com

**Scientific Category: MYELOID NEOPLASIA**
Abstract

Mastocytosis is a heterogeneous disease characterized by the accumulation of mast cells in one or more organs. Our objective was to identify a peripheral mast cell precursor and assess its variation rate in mastocytosis. A peripheral blood phenotypic analysis was performed among 50 patients with mastocytosis who were enrolled in a prospective multicentric French study, and the phenotypic analysis results of the patients were compared with those of healthy donors. The rate of peripheral blood CD34\(^c\)-Kit\(^+\) cells correlated with the severity of mastocytosis. This cellular population was isolated from healthy donors as well as from patients with systemic mastocytosis. After 30 days of culture, the CD34\(^c\)-Kit\(^+\) cells gave birth to mature mast cells, indicating that this cellular population constitutes a mast cell circulating precursor. Monitoring peripheral CD34\(^c\)-Kit\(^+\) cells by flow cytometry could be a useful and low-invasive tool to determine the disease severity, the relapses and to assess treatment efficiency.

Key words: MASTOCYTOSIS, MAST CELL, KIT, PROGNOSIS, TREATMENT.
Introduction

Mastocytosis is a rare heterogeneous disease characterized by the accumulation of mast cells (MC) in one or several organs \(^1\)-\(^6\). A World Health Organization (WHO) classification described several disease subcategories, broadly divided into localized versus systemic disease. Systemic mastocytosis (SM) is subsequently divided into indolent and aggressive disease based on damage to organs \(^6\)-\(^8\). The c-Kit tyrosine kinase receptor is expressed on MCs. Adults with SM usually present \(c\text{-}Kit\) mutations, most frequently D816V, which allows MC survival and proliferation. Recently, flow cytometric studies showed that pathologic MCs from patients with mastocytosis, in addition to c-Kit expression, display unique aberrant immunophenotypic characteristics, as compared to normal MCs \(^9\)-\(^14\). Normal MCs are derived from CD34\(^-\)c-Kit\(^+\) blood precursors, which terminally differentiate in peripheral tissues upon SCF stimulation \(^15\).

The aim of our study was to detect CD34\(^-\)c-Kit\(^+\) cells in peripheral blood (PB) of patients with mastocytosis and investigate whether these cells could give birth to mature MCs in the presence or absence of SCF as well as to investigate the relevance of CD34\(^-\)c-Kit\(^+\) cells detection for positive diagnosis, classification and follow-up.
Patients and methods

Patients and data collection:
Fifty consecutive patients (26 men and 24 women) with a mastocytosis diagnosis as defined by the WHO criteria and 15 healthy donors were enrolled in a prospective national multicentric study from 1999 to 2008. Mastocytosis subcategories comprised cutaneous (n=4), indolent SM (n=25), aggressive SM (n=16) and SM with associated hematological non-MC-lineage disease (SM-AHNMD; n=5). The median age of phenotype was 52 years [7-75]. Three patients (patient 32, 38 and 42) were followed at each hospitalization. A PB phenotypic analysis was performed (see below), and the results were correlated with clinical manifestations and treatment responses. All patients were included in the “Mastocytosis Pathophysiological Study,” sponsored by the Association for the Initiative and Research of Mastocyte and Mastocytosis (AFIRMM), which began in 2003. The study was approved by the Necker Hospital Ethical Committee and was carried out in accordance with the Helsinki convention. Each patient provided an informed consent. The c-Kit gene sequencing, was performed for each patients in skin, blood and/or bone marrow. The D816V mutation of c-Kit was detected among 35 patients, including patients 32, 38, and 42. Among them, thirteen were c-Kit WT (Table).

PBMC isolation, Flow cytometry analysis and cell culture
Blood was collected on heparinized tubes and PBMC were isolated by Ficoll-IPaque gradient (Amersham Life Science, U.K). PBMCs were stained using: CD34 conjugated to fluorescein isothiocyanate (FITC) and CD117/c-Kit to phycoerythrin (PE); Control isotype-matched antibodies were used at appropriate concentration (Beckman Coulter®). After washing, 2x10⁴ events were analysed by FACS-Calibur (Becton Dickinson®). The c-Kit⁺ cells were sorted after c-Kit-APC staining associated to APC-coated magnetic beads (Miltenyi® Biotec®). The c-Kit⁺ sorted cells were stained with CD34-FITC, c-Kit-APC and CD45-PerCP (Beckton Dickinson). The c-Kit⁺CD34⁺CD45low cells were sorted on a BD Aria I® FACS sorter and cultured on 96 well plates in 200 µL of IMDM medium supplemented with or without Stem Cell Factor (SCF) adjunction (50 µg/mL). The medium was renewed twice a week. For each cytospin, 100 µL of PBS 2% FBS containing 10,000 sorted cells were used.
and stained with May Grünwald Giemsa at culture day 0 and 30. The histamine was measured in the supernatant as previously described \(^{17}\).

**D816V c-Kit mutation detection and serum tryptase measurement**

The D816V c-Kit mutation was determined as previously described \(^{18,19}\). Total serum tryptase (protryptase + \(\beta\)-tryptase) was measured using fluorescent enzyme-linked immunoassay (Unicap; Pharmacia) \(^{20}\). The detection limit of this assay is 1 ng/mL, and in healthy controls, serum tryptase levels range between <1 and 15 ng/mL, with a median of ~ 5 ng/mL \(^{21}\).

**Statistical analysis:**

Statistical comparisons between characteristics of healthy donors and patients (CM, ISM, ASM, SM-AHNMD) were based on unpaired t test. All reported p values were two tailed with confidence intervals of 95% and p value <0.05 was considered significant. Data were analyzed using GraphPad Prism software, version 5.01 (GraphPad Software Inc., San Diego, CA).
Results and discussion

The rate of the circulating CD34-c-Kit+ cell population was significantly higher among the SM subcategory patients (all p<0.005) compared to the healthy donors and CM patients (Fig. 1A, 1B). The rate was more elevated among patients with ASM and SM-AHNDM than those with indolent forms of SM. Serum tryptase rate analysis revealed that tryptase was significantly higher in all mastocytosis subcategories (CM p=0.0385; ISM p=0.0242, ASM p=0.0005 and SM-AHNMD p=0.0012). The number of CD34-c-Kit+ cells was assessed during disease evolution and under various treatments of three ASM patients (Fig. 1C, 1D, 1E). These results showed that percentage of CD34-c-Kit+ cells decreased 24 to 48 hours after each efficient treatment and increased after each relapse, preceding serum tryptase level modification. Taken together, these results demonstrate that a circulating CD34-c-Kit+ cell population exists among patients with mastocytosis, and the population rate correlated with mast cell burden, with higher elevation rates among SM subcategories as compared to CM subcategories and healthy donors (p= 0.0146 versus 0.178). This CD34-c-Kit+ cell population was markedly higher among patients with the most aggressive SM subcategories, such as ASM and SM-AHNMD. These more aggressive SM forms are associated with poor prognosis and shortened life expectancy. The CD34-c-Kit+ cell population number correlated to cytoreductive treatment efficiency and could predict clinical disease relapses (Fig. 1C-E).

CD34-c-Kit+ cells were isolated from healthy donors (n=3) and patients (n=4) and cultured in a medium supplemented or not with SCF. Among the patients, three were bearing the D816V c-Kit mutation and one was c-Kit WT. After 30 days of culture in the presence of SCF, mature MCs were identified by morphology and histamine secretion (Fig. 1F). In absence of SCF, only MCs derived from patients with mastocytosis (bearing WT c-Kit or D816V mutation) were able to differentiate into mature MCs, in contrast to healthy donors. This result suggested that the MCs precursor from all patients with mastocytosis could mature, independently from SCF.

Previous studies reporting MC immunophenotype on large patient cohorts were usually performed on bone marrow (BM) MCs, as recommended. Our results on a large patient cohort show that PB-phenotype could be useful, in addition to BM aspiration, to study MC phenotype among patients with mastocytosis. Further, the PB-phenotype could represent an alternative from BM aspiration to follow disease activity or to monitor treatment efficiency, because the result of PB-phenotype is fast, reproducible, and more sensitive than serum tryptase measurement.
In addition, our findings give insight to the pathophysiology of mastocytosis and normal MC lineage development by providing strong evidence that MC accumulation in various tissues originates from the amplification of clonal blood-circulating precursors that are able to differentiate and survive independently from SCF because of constitutive activation of c-Kit as a consequence of c-Kit mutations or other unknown oncogenic events. These data may open new avenues of research in the field of MCs precursors.
Acknowledgments

The authors wish to thank the following physicians for collecting patient data: Drs. M. Blanc (Chambéry), D. Bordessoule (Limoges), O. Fain (Bondy), C. Hoarau (Tours), H. Coignard (Paris), F. Suarez (Paris) and C. Roux-Serratrice (Marseille). We are also grateful to François Machavoine, Fabienne Palmérini, Elke Schneider and Françoise Valensi for technical assistance and helpful discussions.

Fundings

S. G-L is a recipient from a grant from the Fondation pour la Recherche médicale (FRM) and SB is a recipient from a grant from Société Française de dermatologie. This work was partially financed by INSERM, la Ligue Nationale Contre le Cancer (Equipe labellisée PD and OH) and ANR-MRAR (Agence Nationale pour la Recherche, grant Maladies Rares – PD and OH), FRM (PD and OH) and Inca (Institut National du Cancer, grant translationnel PD and OH).

Author contributions

Contributions:
S. G-L wrote the manuscript, carried out experiments, collected data, analyzed and interpreted data.
Y.L designed the study, carried out experiments, collected, analyzed, interpreted data, and assisted in writing the manuscript.
O.H designed the study, interpreted data, recruited patients, assisted in writing and finalised the manuscript.
P.D, F.F, S.B carried out experiments, collected data, analyzed, interpreted data and assisted in writing the manuscript.
C.B, K.H, L.L, carried out experiments, collected and analyzed data.
M-O. C, J-M. L, C de G, G.D, F.L, M.H and O.L, helped design the study, edited the manuscript and recruited patients.

Competing interests

The authors declare no competing interest
Abbreviations

ASM: Aggressive systemic mastocytosis
CM: Cutaneous mastocytosis
FBS: foetal bovine serum
ISM: Indolent Systemic mastocytosis
MCs: Mast cell
MCL: Mast cell leukaemia
MCS: Mast cell sarcoma
PB: Peripheral blood
PBMC: Peripheral blood mononuclear cell
PBS: phosphate-buffered saline
SCF: Stem cell factor
SM: Systemic mastocytosis
SM-AHNMD: SM with an associated clonal haematological non-MC-lineage disease
References


<table>
<thead>
<tr>
<th>Patient's number</th>
<th>Age at phenotype</th>
<th>Tryptase (μg/L)</th>
<th>Valent stage</th>
<th>c-Kit Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>20</td>
<td>CM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>5</td>
<td>CM</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>1</td>
<td>CM</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>5</td>
<td>CM</td>
<td>WT</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>45</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>50</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>7</td>
<td>67</td>
<td>9</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>22</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>9</td>
<td>75</td>
<td>124</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>153</td>
<td>ISM</td>
<td>WT</td>
</tr>
<tr>
<td>11</td>
<td>52</td>
<td>93</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>12</td>
<td>51</td>
<td>21</td>
<td>ISM</td>
<td>WT</td>
</tr>
<tr>
<td>13</td>
<td>65</td>
<td>318</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>14</td>
<td>49</td>
<td>40</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>15</td>
<td>43</td>
<td>16</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>16</td>
<td>60</td>
<td>140</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>17</td>
<td>75</td>
<td>2</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>18</td>
<td>57</td>
<td>48</td>
<td>ISM</td>
<td>WT</td>
</tr>
<tr>
<td>19</td>
<td>37</td>
<td>11</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>20</td>
<td>37</td>
<td>50</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>21</td>
<td>30</td>
<td>49</td>
<td>ISM</td>
<td>WT</td>
</tr>
<tr>
<td>22</td>
<td>55</td>
<td>229</td>
<td>ISM</td>
<td>WT</td>
</tr>
<tr>
<td>23</td>
<td>23</td>
<td>100</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>24</td>
<td>39</td>
<td>6</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>25</td>
<td>72</td>
<td>822</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>26</td>
<td>18</td>
<td>28</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>27</td>
<td>62</td>
<td>33</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>28</td>
<td>30</td>
<td>68</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>29</td>
<td>26</td>
<td>152</td>
<td>ISM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>30</td>
<td>45</td>
<td>17</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>31</td>
<td>38</td>
<td>233</td>
<td>ASM</td>
<td>WT</td>
</tr>
<tr>
<td>32</td>
<td>70</td>
<td>421</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>33</td>
<td>72</td>
<td>304</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>34</td>
<td>25</td>
<td>228</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>35</td>
<td>36</td>
<td>49</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>36</td>
<td>7</td>
<td>734</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>37</td>
<td>42</td>
<td>15</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>38</td>
<td>68</td>
<td>304</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>39</td>
<td>70</td>
<td>330</td>
<td>ASM</td>
<td>WT</td>
</tr>
<tr>
<td>40</td>
<td>43</td>
<td>3</td>
<td>ASM</td>
<td>WT</td>
</tr>
<tr>
<td>41</td>
<td>74</td>
<td>156</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>42</td>
<td>49</td>
<td>39</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>43</td>
<td>55</td>
<td>95</td>
<td>ASM</td>
<td>WT</td>
</tr>
<tr>
<td>44</td>
<td>64</td>
<td>5</td>
<td>ASM</td>
<td>nd</td>
</tr>
<tr>
<td>45</td>
<td>32</td>
<td>356</td>
<td>ASM</td>
<td>D-816-V</td>
</tr>
<tr>
<td>46</td>
<td>72</td>
<td>200</td>
<td>SM-AHNMD</td>
<td>D-816-V</td>
</tr>
<tr>
<td>47</td>
<td>52</td>
<td>62</td>
<td>SM-AHNMD</td>
<td>D-816-V</td>
</tr>
<tr>
<td>48</td>
<td>54</td>
<td>12</td>
<td>SM-AHNMD</td>
<td>WT</td>
</tr>
<tr>
<td>49</td>
<td>40</td>
<td>10</td>
<td>SM-AHNMD</td>
<td>D-816-V</td>
</tr>
<tr>
<td>50</td>
<td>73</td>
<td>98</td>
<td>SM-AHNMD</td>
<td>D-816-V</td>
</tr>
</tbody>
</table>
Figure legends:

Figure 1: (A, B) Correlation of Valent’s stage disease with the rate of circulating CD34\(^{-}\)c-Kit\(^{+}\) population and the rate of serum tryptase. (A) The rate of the circulating CD34\(^{-}\)c-Kit\(^{+}\) is shown for each patient along with their disease stage, indicating the aggressiveness of their disease, and compared to healthy controls. All mastocytosis patients with systemic forms had a significantly higher rate of CD34\(^{-}\)c-Kit\(^{+}\) cells than the controls: ISM (p=0.0007); ASM (p=0.0031) and SM-AHNMD (p=0.0005). This association was not found for cutaneous forms, which were comparable to the healthy controls (p=0.5). (B) The serum rate of tryptase was always elevated among patients with mastocytosis, independently from the stage of the disease: cutaneous as well as systemic forms had an elevated serum tryptase. Indeed, serum tryptase levels among controls were always lower than patients with CM (p=0.0385), ISM (p=0.0242), ASM (p=0.0005) or SM-AHNMD (p=0.0012). (CM: cutaneous mastocytosis; SM: systemic mastocytosis; ISM: Indolent SM; ASM: aggressive SM; and SM-AHNDM: SM associated with hematological non-mast cell disease). (C, D, E). Clinical and biological follow-up of three patients with aggressive systemic mastocytosis until their death. On each panel (C: Patient 42; D: Patient 32; E: Patient 38) the clinical evolution and treatment is mentioned as well as the rate of the circulating CD34\(^{-}\)c-Kit\(^{+}\) population. It shows that when patients present aggressive disease with massive mast cell infiltration, the rate of the circulating CD34\(^{-}\)c-Kit\(^{+}\) population is a good tool to quickly follow (within 24-48 hours) the clinical evolution of the disease and to determine the efficiency of the treatments. (CT: healthy control; 2CDA: Leustatine, Zenapax, Daclizumab; C1 to C4: cure number).

Figure 2: Cytological aspects (MGG smears) and histamine production of isolated CD34\(^{+}\)/c-Kit\(^{+}\) compartments from peripheral blood of patients with mastocytosis and healthy controls. (A) Among healthy donors, cultures of both CD34\(^{+}\)c-Kit\(^{+}\) and CD34\(^{-}\)c-Kit\(^{+}\) cells subsets showed differentiation into mature mast cells in the presence of SCF (upper panel), whereas no differentiation was observed in the absence of SCF (data not shown). In patients with proven mastocytosis, bearing either the classical D816V c-Kit mutation or WT c-Kit, similar differentiation into mature mast cells was observed in the presence or absence of SCF (lower panel). (B) Histamine dosage also supported mast cell differentiation, demonstrating increased histamine levels with or without SCF in both cultured subsets from the patients with mastocytosis, whereas among healthy controls, histamine levels increased only in the presence of SCF.
Figure 2

A

Healthy Donor (SCF+)

CD34-Kit+ CD34+Kit+

Mastocytosis c-Kit D816V

Mastocytosis c-Kit WT

B

Histamine level

ng/ml

Day 0 SCF- SCF+

Day 30

Histamine level

ng/ml

Day 0 SCF- SCF+

Day 30

Histamine level

ng/ml

Day 0 SCF- SCF+

Day 30

Histamine level

ng/ml

Day 0 SCF- SCF+

Day 30
Blood CD34⁻c-Kit⁺ cell rate correlates with aggressive forms of systemic mastocytosis and behaves like a mast cell precursor

Sophie Georgin-Lavialle, Ludovic Lhermitte, Cédric Baude, Stéphane Barete, Julie Bruneau, Jean-Marie Launay, Marie-Olivia Chandesris, Katia Hanssens, Christian de Gennes, Gandhi Damaj, Fanny Lanternier, Mohamed Hamidou, Olivier Lortholary, Patrice Dubreuil, Frédéric Feger, Yves Lepelletier and Olivier Hermine