

Mast cells from different molecular and prognostic subtypes of systemic mastocytosis display distinct immunophenotypes

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Background: Systemic mastocytosis (SM) is a heterogeneous group of disorders with distinct clinical and biological behavior. Despite this, little is known about the immunophenotypic features of the distinct diagnostic categories of SM.

Objective: To analyze the immunophenotypic characteristics of bone marrow (BM) mast cells (MCs) of different subtypes of SM.

Methods: Bone marrow samples from 123 patients with different subtypes of SM and 92 controls were analyzed for a broad panel of immunophenotypic markers by flow cytometry.

Results: Three clearly different maturation-associated immunophenotypic profiles were found for BMMCs in SM. These different profiles were associated with both genetic markers of the disease and its clinical behavior. BMMCs from poor-prognosis categories of SM (aggressive SM and MC leukemia) typically showed an immature phenotype with clonal

involvement of all myeloid lineages by the D816V stem cell growth factor receptor gene (*KIT*) mutation. In turn, a mature activated versus resting BMMC immunophenotype was commonly found among patients with good-prognosis subtypes of SM depending on whether they carried (indolent SM and clonal MC activation disorders) or not (well differentiated SM) the D816V *KIT* mutation.

Conclusion: Bone marrow MCs from SM show 3 different maturation-related immunophenotypic profiles that are associated with both the genetic markers of the disease and its clinical behavior. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Mastocytosis, immunophenotype, flow cytometry, *KIT* mutations

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Mastocytosis is a heterogeneous group of clonal mast cell (MC) disorders characterized by abnormal proliferation and accumulation of MCs in 1 or multiple tissues.¹ Most frequently, clonally expanded MCs carry the D816V or other activating stem cell growth factor receptor gene (*KIT*) mutations, which translate into morphologic atypia,²⁻⁴ functional transformation,⁵ and an aberrant immunophenotype.⁶ In fact, bone marrow (BM) MCs from systemic mastocytosis (SM) typically exhibit unique immunophenotypic features, and aberrant expression of CD25 and/or CD27 is used as a minor diagnostic criterion for SM.^{1,8} In addition to CD25 and CD2 expression, BMMCs from SM commonly show other aberrancies such as overexpression of the CD63⁹ and CD69¹⁰ activation molecules, CD58—a ligand for the CD2 protein¹¹—CD33,⁷ and several complement-associated molecules—for example, CD11c, CD35, CD59, and CD88.^{6,12} In contrast, expression of kit (CD117),¹¹ the CD71 transferrin receptor, and the CD29 β 1-integrin are abnormally downregulated.⁷

Previous reports suggest that such phenotypic changes of BMMCs in SM could reflect MC activation because of constitutive activating *KIT* mutations.^{6,13} In line with this, recent studies indicate that >90% of all patients with SM carry the D816V *KIT* mutation.¹⁴ However, currently it is well established that SM is not a uniform disease and that it includes several clinicopathological entities and subvariants with different outcomes.^{1,8,15-19} The clinicopathological and prognostic heterogeneity of SM suggests that some patients with SM might carry genetic lesions in addition to the *KIT* mutation (eg, constitutive activation of ras-related protein m-ras^{20,21}), which could contribute to explaining the variable immunophenotypic patterns and interactions of MCs with their microenvironment, similar to what has been demonstrated for

Abbreviations used

ASM:	Aggressive systemic mastocytosis
ASM-AHNMD:	Aggressive systemic mastocytosis associated with a clonal non-mast cell lineage hematopoietic disease
BM:	Bone marrow
cMCAD:	Clonal mast cell activation disorder
CPA:	Carboxypeptidase A
CyB12:	Cytoplasmic total tryptase
CyG5:	Cytoplasmic mature tryptase
FDR:	False discovery rate
ISM:	Indolent systemic mastocytosis
ISM-AHNMD:	Indolent systemic mastocytosis associated with a clonal non-mast cell lineage hematopoietic disease
MC:	Mast cell
MCL:	Mast cell leukemia
NPV:	Negative predictive value
PPV:	Positive predictive value
SM:	Systemic mastocytosis
SM-AHNMD:	Systemic mastocytosis associated with a clonal non-mast cell lineage hematopoietic disease
SSC:	Sideward light scatter
sT:	Serum tryptase
TN:	True negative
TP:	True positive
WDSM:	Well differentiated systemic mastocytosis

other hematologic malignancies.²²⁻²⁴ Despite this, current knowledge about the phenotypic features of different subtypes of SM is rather limited because most phenotypic studies have either focused on specific subtypes of mastocytosis—for example, indolent SM (ISM)^{7,9,10}—or analyzed a relatively limited number of molecules in relatively restricted cohorts of patients.^{25,26} Furthermore, no relationship between the MC phenotype and the distinct subtypes of SM has been investigated in detail so far. Interestingly, preliminary results^{14,16,17} suggest that the pattern of expression of CD2 and CD25 by BMMCs from well differentiated SM (WDSM)—a recently described variant of SM that frequently lacks D816V *KIT* mutation—could differ from other subtypes of mastocytosis.¹⁴ These findings would further support the existence of a genotypic/phenotypic association among SM.

Here, we analyzed the immunophenotype of BMMCs from a series of 123 patients with SM and compared it among individuals with different subtypes of the disease, as well as with presence or absence of the D816V *KIT* mutation. Our results show that BMMCs from SM are phenotypically heterogeneous with 3 clearly different profiles that are associated with molecular and prognostic subtypes of mastocytosis.

METHODS**Patients, controls, and samples**

A total of 215 BM samples were obtained from adult individuals, including 123 patients (66 men and 57 women; median age, 45 years; range, 19–83 years) consecutively diagnosed with SM²⁷ at the reference centers of the Spanish Network on Mastocytosis (REMA; Mast Cell Unit, Hospital Virgen del Valle, Toledo; and Cytometry Service, Cancer Research Centre, Salamanca, Spain)^{1,8} and 92 normal BM donors, which included 40 normal subjects and 52 patients undergoing BM aspiration for clinical reasons other than mastocytosis (see this article's Table E1 in the Online Repository at www.jacionline.org). In all cases, informed consent was given by each individual before the study, according to the guidelines of the local Ethical Committees.

According to the World Health Organization criteria,^{1,8} patients with SM were classified as follows: ISM, 69 cases; aggressive SM (ASM), 9; MC leukemia (MCL), 3; and SM associated with a clonal non-MC lineage hematopoietic disease (SM-AHNMD), 14 patients—8 had ISM (ISM-AHNMD), and 6 had ASM (ASM-AHNMD). The other 28 patients with SM corresponded to 2 recently described subvariants of SM: clonal MC activation disorder (cMCAD)^{18,19} (n = 17) and WDSM^{16,17} (n = 11).

Multiparameter flow-cytometry immunophenotypic studies of BMMCs

Multiparameter flow-cytometry immunophenotypic studies were performed on BM aspirate samples collected in Vacutainer tubes containing lithium heparin (Becton/Dickinson—BD-Labware, Franklin Lakes, NJ). All samples were processed within the first 24 hours after they were collected. For sample preparation, a direct immunofluorescence stain-and-then-lyse technique was used, as described elsewhere.⁹ The expression of cytoplasmic markers was evaluated after staining for surface antigens by using the FIX & PERM reagent kit (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Four-color combinations of mAbs were used to stain BM cells with a broad set of reagents (see this article's Table E2 in the Online Repository at www.jacionline.org). For each sample, data acquisition was performed in 2 steps in a FACSCalibur flow cytometer (BD Biosciences) with the CellQUEST software (BD Biosciences) as previously described in detail.⁷ For data analysis, both the INFINICYT (Cytognos SL, Salamanca, Spain) and the Paint-A-Gate PRO (BD Biosciences) software programs were used.

Detection of *KIT* mutation

KIT mutation—D816V or other mutations localized at codons 814 to 819 (exon 17)—was detected on highly purified ($\geq 97\%$ purity) BM cell populations as previously described.^{14,28} In turn, identification of *KIT* mutations at exon 11 was performed on genomic DNA by direct sequencing of the amplified PCR products in both directions, using the dye-deoxy terminator method, in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif) and both the 5'-CCA GAG TGC TCT AAT GAC TG-3' and 5'-AGC CCC TGT TTC ATA CTG AC-3' primers (Isogen Life Sciences, Maarsen, The Netherlands).

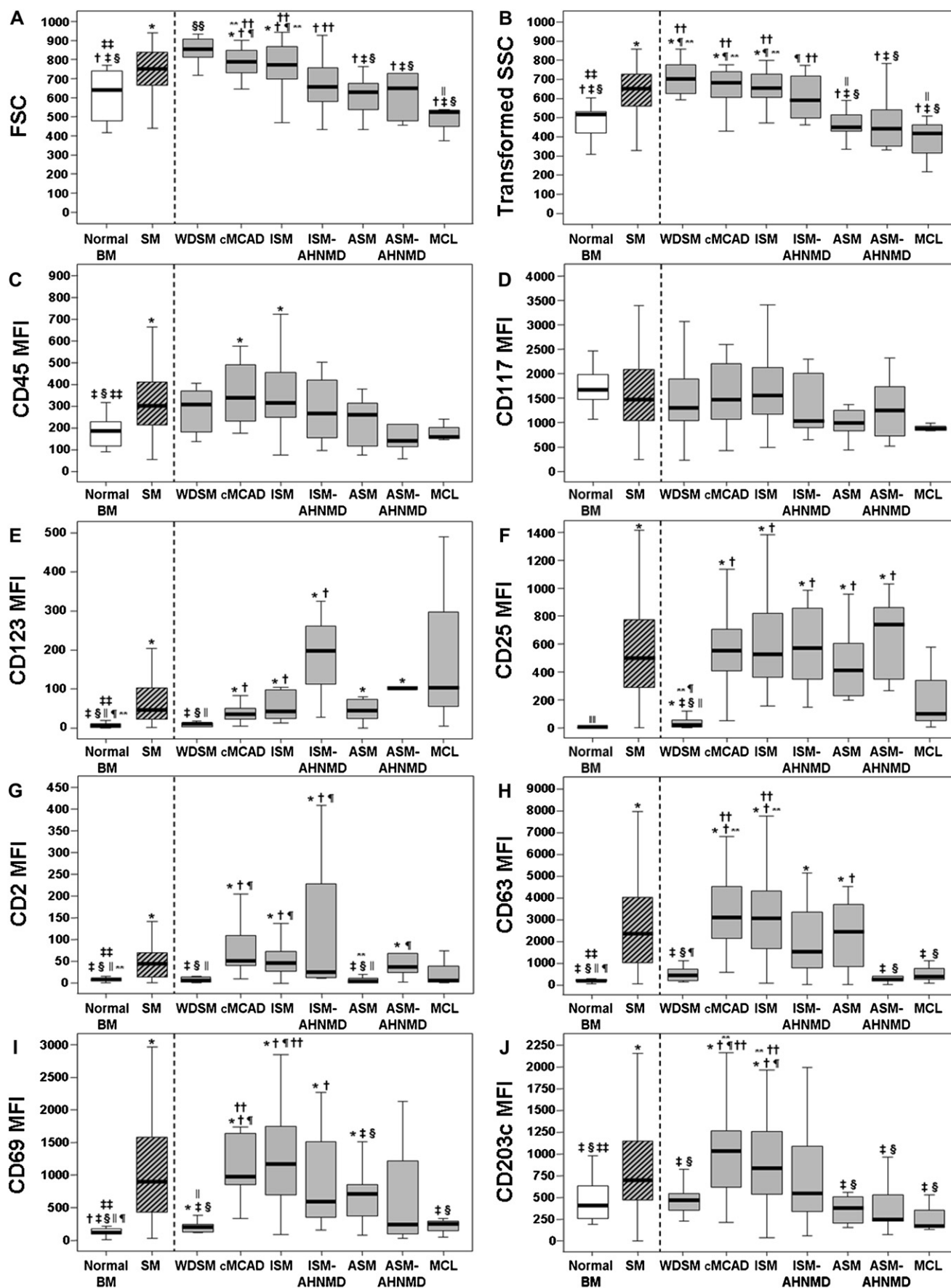
Serum tryptase levels

Serum tryptase (sT) levels were determined by using a commercially available standard ELISA technique (Phadia ImmunoCAP Tryptase System; Phadia, Uppsala, Sweden), following the manufacturer's instructions.

Statistical methods

For all continuous variables, median, mean, and SD values, as well as range and the 25th and 75th and the 10th and 90th percentiles, were calculated; for categorical variables, frequencies were reported. Comparisons between groups were performed with either the nonparametric Kruskal-Wallis and Mann-Whitney *U* tests (for continuous variables) or the Pearson χ^2 and Fisher exact tests (for categorical variables); a linear regression model was used to explore the degree of correlation between different variables (SPSS 15.0 software, Chicago, Ill). *P* values $< .05$, with a false discovery rate (FDR) correction for multiple comparisons of $< 10\%$, were considered to be associated with statistical significance.

To classify each case according to the immunophenotypic features of BMMCs, a score was built based on the expression of individual markers. For this purpose, phenotypes were considered aberrant if their divergence from normal/reactive BMMCs was $> \text{mean} \pm 1 \text{ SD}$ and a score of 0 or 1 was given when the MFI of individual markers was $\leq \text{mean} \pm 2 \text{ SD}$ of the mean value obtained for that particular marker depending on whether it was different or coincident with the expression described for the specific diagnostic groups (ISM/cMCAD, WDSM, or ASM/MCL), respectively; a score of 2 and 3 was assigned when the MFI value divergence was $> \text{mean} \pm 2 \text{ SD}$ but $< \text{mean} \pm 3 \text{ SD}$ and when it was $> \text{mean} \pm 3 \text{ SD}$ of the values observed for the



corresponding disease category, respectively. Sensitivity was calculated as true positive (TP)/(TP + false negative), specificity as true negative (TN)/(TN + false positive), positive predictive value (PPV) as TP/(TP + false positive), and negative predictive value (NPV) as TN/(TN + false negative). Receiver operating characteristic curves were used to assess the sensitivity and specificity of immunophenotyping for the diagnosis and classification of SM and its 3 phenotypic subtypes.

RESULTS

Flow-cytometric pattern of BM infiltration by MCs

Patients with SM displayed increased BMMC counts (mean $\pm$ 1 SD) versus normal BM ($1.7\% \pm 6.8\%$ vs $0.07\% \pm 0.11\%$; $P < .0001$). ASM, MCL, and SM-AHNMD showed significantly higher BMMC counts than cMCAD and ISM ($P < .05$; Table E1).

Immunophenotypic characteristics of normal/reactive BMMCs

Normal/reactive (control) BMMCs displayed relatively high light scatter values and expressed the CD45, CD117, CD63, CD203c, CD59, FcεRI, CD32, HLA-I, cytoplasmic carboxypeptidase, and cytoplasmic total tryptase (CyB12; Figs 1-3) markers in the absence of reactivity for CD2, CD25, CD123 (Fig 1), and CD34 (not shown). Reactivity for CD64, CD16, HLA-DQ, and HLA-DR (Fig 2) was variable and only detected (partially or dimly expressed) in a restricted number of individuals—5%, 13%, 13%, and 25%, respectively. In turn, CD22 tested positive in half of the controls, with extremely variable—dim to strong—patterns of expression (Fig 2). Mature tryptase (CyG5), CD69, and cytoplasmic b-cell lymphoma 2 protein (CyBcl2) were expressed in the majority of control BM samples—75%, 88%, and 94%, respectively (Figs 1-3). This was associated with relatively high amounts of immature tryptase (high CyB12/CyG5 ratio), low total sT/CyB12 values, and relatively high levels of sT per BMMC (high sT/BMMC ratio; Fig 3).

Overall immunophenotypic features of BMMCs in SM

In comparison with normal BMMCs, clonal BMMCs from patients with SM showed similar patterns of expression of CD117^{hi}, FcεRI⁺, HLA-I⁺, and CD34⁺, except for 1 patient with MCL who expressed CD34 in 28% of the pathological MC. Conversely, they displayed higher light scatter values ($P < .01$), and aberrant expression of CD25^{hi}, CD2, and CD123 in 93%, 72%, and 73% of cases, respectively ($P \leq .0001$; Fig 1). Similarly, expression of CD22, HLA-DR, CD64, CD16, and HLA-DQ, was abnormally increased on BMMCs from most SM: 96%, 85%, 84%, 69%, and 58% of cases, respectively ($P \leq .02$; Fig 2). Reactivity for the CD69, CD63, and CD203c activation markers, FcγRII (CD32), CD45 and the CD59 complement regulatory protein, was also abnormally high in SM ($P < .05$; Figs 1 and 2). In addition, BMMCs from patients with SM showed abnormally low expression of CyB12 with both increased cytoplasmic expression of mature tryptase (CyG5) and higher sT

values ($P = .02$), leading to a decreased serum tryptase/BMMC ratio ($P = .01$) and an increased sT/CyB12 ratio (Fig 3).

Aberrant marker expression allowed for a clear discrimination between normal and SM BMMCs, with a sensitivity of 98.1% and a specificity of 100% (PPV, 100%; NPV, 80%).

Differential immunophenotypic profiles of BMMCs from patients with different subtypes of SM

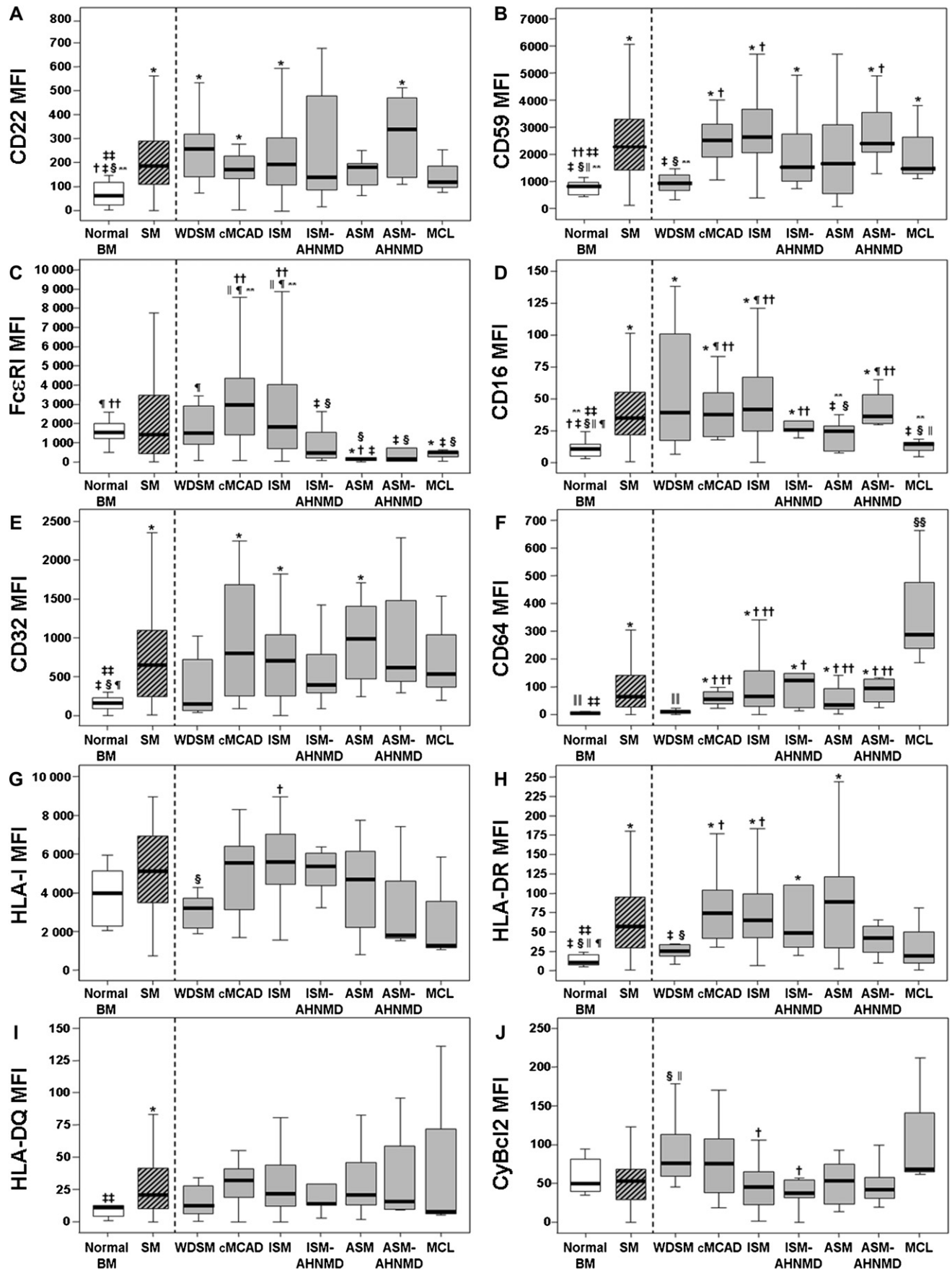
Overall, 3 clearly distinct immunophenotypic profiles were found among SM (see this article's Table E3 in the Online Repository at www.jacionline.org), which corresponded to patients with (1) WDSM, (2) ISM/cMCAD, and (3) both ASM and MCL; SM-AHNMD cases showed a heterogeneous and variable immunophenotype depending on whether the SM component corresponded to ISM or ASM.

Patients with ISM and cMCAD displayed similar immunophenotypic patterns, with aberrantly increased light scatter ($P \leq .003$ vs control BMMCs) and uniform CD25^{hi} expression ($P < .0001$); most cases were also CD2⁺ (88% and 81%, respectively; $P < .0001$; Fig 1). Moreover, BMMCs from cMCAD and ISM showed increased expression of CD16 and CD45 ($P < .005$) and very high reactivity ($P \leq .01$) for CD59, CD63, CD69, CD203c, CD32, CD64, CD123, and HLA-DR (Figs 1 and 2). Although patients with ISM and cMCAD displayed overall increased sT levels ($P < .04$), this was associated with decreased sT levels per BMMC for ISM ($P = .02$; Fig 3); in both groups of patients with SM, higher sT was associated with a decreased CyB12 (total cytoplasmic tryptase).

In contrast with ISM and cMCAD, BMMCs from most patients with WDSM were CD25⁺ and CD2⁺ ($P < .0001$), with only 4 of 11 cases either partially positive for CD25 ($n = 3$) or CD2^{dim}/CD25⁺ ($n = 1$). In addition, BMMCs from WDSM exhibited abnormally increased light scatter ($P \leq .006$ vs control BMMCs; Fig 1), and expression of CD16, CD22 ($P = .003$ vs control BMMCs), CyBcl2 ($P = .006$ vs ISM), cytoplasmic carboxypeptidase, and CyB12 ($P = .003$ vs ISM; Figs 1-3). The greater expression of CyB12 was associated with relatively low sT and a decreased sT/BMMC ratio ($P \leq .008$ vs control subjects, cMCAD, and ISM) but normal total (CyB12)/mature (CyG5) cytoplasmic tryptase levels (Fig 3).

In turn, poor-prognosis SM (ASM and MCL) typically showed an aberrant CD25⁺ (9/9 ASM and 2/3 MCL; $P \leq .001$ vs control BMMCs) but CD2⁺ phenotype with only 1 of 9 patients with ASM and 1 of 3 patients with MCL CD2^{dim} ($P < .0001$ for ASM vs ISM and cMCAD). Likewise, expression of CD63 and CD69 was significantly increased among patients with ASM ($P \leq .02$ vs normal BM) but not patients with MCL (Fig 1). Furthermore BMMCs from ASM, ASM-AHNMD, and MCL, showed aberrantly low light scatter properties—especially sideward light scatter (SSC; $P \leq .02$ vs good-prognosis SM)—along with decreased CD117 and FcεRI expression ($P \leq .03$ vs normal BM). Other markers, such as CD59 ($P = .02$ for MCL), HLA-DR, CD123, and CD32 ($P < .02$ for ASM), were frequently overexpressed versus controls. Similarly, poor-prognosis variants of SM displayed the

FIG 1. Light scatter (forward light scatter (FSC), **A**; sideward light scatter (SSC), **B**) and immunophenotypic characteristics (**C-J**) of BMMCs from adults with different subtypes of SM versus normal/reactive BMMCs, gated as CD117^{hi}/CD45⁺ with intermediate-to-high scatter. MFI, Mean fluorescence intensity/BMMC. $P < .05$ and FDR 10% versus *controls, †WDSM, ‡cMCAD, §ISM, ||ISM-AHNMD, ¶ASM, **ASM-AHNMD, ††MCL, ‡‡SM, §§all other groups except SM, and ||||all other groups except SM and WDSM.



highest sT levels ($P \leq .03$ vs good-prognosis SM; Table E1) in association with decreased CyB12 expression leading to an increased sT/CyB12 ratio and decreased sT levels per BMMC (Fig 3).

As described, ISM-AHNMD and ASM-AHNMD displayed similar phenotypic profiles to their ISM and ASM non-associated with a clonal non-mast cell lineage hematopoietic disease counterparts, except for CD117, FcεRI, CyB12, and CD123, whose expression in ISM-AHNMD was closer to that observed among ASM than to ISM (Figs 1-3). Regarding ASM-AHNMD, phenotypic features intermediate between those of ASM and MCL were found for most of the parameters studied, except for CD2 ($P = .04$), CD16 ($P = .03$), and CyG5, which showed a higher and more heterogeneous expression in ASM-AHNMD versus ASM (Figs 1-3).

Based on the expression of individual phenotypic markers, prediction of the specific subtype of SM (WDSM vs ISM/cMCAD vs ASM/MCL) could be achieved with a high sensitivity (67%, 86%, and 100%, respectively) and specificity (100%, 86%, and 88%, respectively; PPV, 100%, 94%, and 62%, respectively; NPV, 96%, 71%, and 100%, respectively). Associations between the immunophenotype of clonal BMMCs from the distinct subtypes of SM and the clinical features of the disease (sT levels, presence of hepatomegaly and/or splenomegaly) have also been found (see the Online Results section of this article in the Online Repository at www.jacionline.org).

Frequency and impact of *KIT* mutations on the immunophenotype of BMMCs in SM

Most patients with SM analyzed (97/109; 89%) displayed *KIT* mutations, except for WDSM, for which only 3 of 10 cases carried the *KIT* mutation (the D816V mutation was positive in only 1 of these patients; $P < .05$ vs all SM variants except MCL; Table E1). Interestingly, the frequency of cases carrying *KIT* mutation restricted to the MC compartment was significantly higher in the good-prognosis (ISM and cMCAD) versus poor-prognosis variants (ASM, MCL, or SM-AHNMD; $P < .05$ for ASM vs ISM and cMCAD and, ASM-AHNMD vs cMCAD), which also displayed *KIT* mutation in other nucleated BM myeloid cells (Table E1).

Among those patients who displayed *KIT* mutations, 95% carried the D816V *KIT* mutation whereas other *KIT* mutations were detected in isolated cases (D816Y, I817V, V819Y, and V560G along with a V815-816 insertion, corresponding to a patient with MCL, a patient with WDSM, and 2 patients with cMCAD, respectively). Overall, no significant phenotypic differences ($P > .05$) were found within each subtype of SM for D816V+ BMMCs versus BMMCs showing either other mutations in the activating loop of *KIT* ($n = 4$) or no *KIT* mutational changes at the loci examined ($n = 6$). However, the only patient with cMCAD showing the V560G *KIT* juxtamembrane mutation displayed unique phenotypic features—CD25^{dim}, CyBcl2^{hi}, CD2⁺, and HLA-I^{dim}—versus other cMCAD cases.

DISCUSSION

Systemic mastocytosis is a clinically and prognostically heterogeneous group of disorders^{1,8,16-19} characterized by the clonal expansion of immunophenotypically aberrant MCs in the patients' BM.^{6,7,9-11} However, little is known about the specific immunophenotypic features of the distinct variants of SM. Here, we analyzed the expression of a broad panel of functional proteins on BMMCs from a large cohort of patients with SM compared with normal/reactive BMMCs. Overall, aberrant phenotypes were identified in all patients with SM, with 3 clearly distinct profiles typically associated with (1) the most common good-prognosis categories of SM (cMCAD and ISM), (2) WDSM, and (3) cases with poor-prognosis subtypes of SM (ASM and MCL).

Currently, aberrant expression of CD25 and/or CD2 on BMMCs represents the only immunophenotypic criterion used in the diagnostic work-up of SM.^{1,8} BMMCs from both cMCAD and ISM showed a typically CD25⁺/CD2⁺ aberrant, mature (eg, FcεRI^{hi}) phenotype associated with overexpression of the CD63, CD69, and CD203c activation markers and the CD64 high-affinity IgG Fc receptor (FcγRI). Interestingly, CD64 is normally absent in resting BMMCs²⁹ but is expressed upon cytokine exposure (INF-γ).^{30,31} Similarly, BMMCs from patients with cMCAD and ISM also showed increased expression of MHC class II molecules (HLA-DR and HLA-DQ) that are typically negative in resting mouse and human MCs but upregulated on activated MCs isolated from tissues infected with pathogens and/or stimulated with cytokines—for example, TNF-α and INF-γ—and LPS.³²⁻³⁴ Altogether, these results suggest that BMMCs from patients with cMCAD and ISM display a phenotypic profile similar to that of activated mature MCs, with aberrant expression of CD2 and CD25. Because virtually every patient within these subgroups of SM carried the D816V *KIT* mutation,¹⁴ which leads to constitutive activation of kit,¹³ this mutation could be responsible for the aberrant activated phenotype of BMMCs in both groups of SM. This hypothesis would be further supported by the absence of phenotypic differences between BMMCs from patients with cMCAD with the D816V *KIT* mutation versus other mutations in the tyrosine kinase loop domain of *KIT*, whereas the only (cMCAD) patient carrying the V560G mutation in the juxtamembrane domain displayed a clearly different immunophenotypic profile.

In contrast with ISM and cMCAD, BMMCs from several other subtypes of SM did not show a CD25⁺/CD2⁺ phenotype. Thus, BMMCs from WDSM were typically CD25⁺/CD2⁺, as previously reported in individual cases¹⁶ and small groups of patients.¹⁷ Furthermore, BMMCs from WDSM also showed normal expression of the CD59, CD203c, and/or CD63 activation markers, which are typically overexpressed by BMMCs from other subgroups of SM.^{6,9,10,12} In fact, BMMCs from WDSM showed a phenotype similar to that of normal resting mature BMMCs, with strong expression of CD117 and FcεRI.^{6,35} In turn, aberrant phenotypes expressed by WDSM were restricted to a few number of cytoplasmic antigens (eg, CyBcl2, carboxypeptidase A [CPA], and tryptase). The increased expression of CyBcl2 (Fig 3) could reflect an altered regulation of apoptosis associated with

FIG 2. Expression of (mean fluorescence intensity [MFI]/BMMC) CD22 (A), CD59 (B), FcεRI (C), FcγR receptors (D-F), HLA molecules (G-I), and CyBcl2 (J) on BMMCs from adults with different subtypes of SM versus normal/reactive BMMCs (CD117^{hi}/CD45⁺ with intermediate-to-high scatter cells). $P < .05$ and FDR 10% versus *controls, †WDSM, ‡cMCAD, §ISM, ||ISM-AHNMD, ¶ASM, **ASM-AHNMD, ††MCL, ‡‡SM, §§all other groups except SM and ISM-AHNMD, and ||||all the other groups except SM and WDSM.

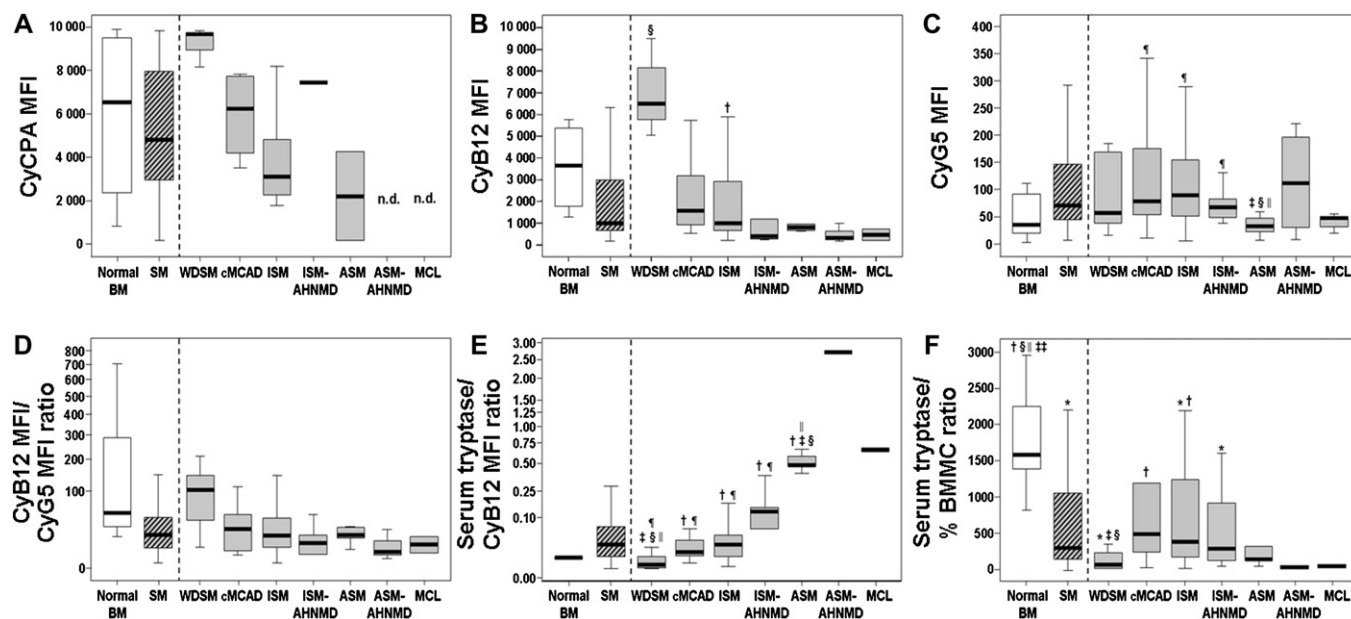


FIG 3. Serum tryptase levels ($\mu\text{g/L}$) and cytoplasmic expression of MC enzymes (mean fluorescence intensity [MFI]) per BMMC ($\text{CD}117^{\text{hi}}/\text{CD}45^+$ with intermediate-to-high scatter cells) from patients with SM versus normal/reactive BMMCs. **A-C**, Expression of cytoplasmic carboxypeptidase A (CPA), total (B12) and mature (G5) tryptase per BMMC. **D**, The ratio between the mean amount of total/mature tryptase per BMMC. **E and F**, The ratio between sT and both CyB12 per BMMC and the percentage of BMMCs, respectively. $P < .05$ and FDR 10% versus *controls, †WDSM, ‡cMCAD, §ISM, ||ISM-AHNMD, ¶ASM, and †‡SM. ND, Not done.

increased survival of MCs³⁶ in WDSM, as previously suggested for cutaneous mastocytosis.³⁷ In turn, the greater amount of cytoplasmic enzymes (eg, tryptase and CPA) could contribute to the typical hypergranulated morphologic appearance and the abnormally increased SSC of BMMCs in WDSM. Overexpression of cytoplasmic tryptase (CyB12) in association with relatively low sT could reflect an impaired secretion phenotype with a significantly decreased release of tryptase per BMMCs in WDSM versus normal BM. Of note, the overall increased cytoplasmic levels of tryptase—pro and mature α/β -tryptase identified by the B12 mAb—detected in WDSM were associated with a normal total/mature tryptase ratio (CyB12/CyG5). Altogether, these results suggest that spontaneous secretion of protryptase could be affected in these patients, because protryptase is spontaneously secreted whereas mature tryptase is stored in granules and released in response to MC stimulation.³⁸ Furthermore, because most patients with WDSM did not show *KIT* mutation and those few patients carrying *KIT* mutations displayed a phenotype similar to that of the nonmutated cases, it could be speculated that BMMCs from WDSM may carry additional mutations/genetic changes involving other proteins downstream of kit, which could be responsible for the impaired MC secretion phenotype.

In contrast with other subgroups of SM, the poor-prognosis variants (ASM and MCL) displayed aberrant positivity for CD25, usually in the absence of CD2. This aberrant phenotype was associated with decreased expression of CD117, Fc ϵ RI, and HLA-I and increased positivity for CD123, HLA-DQ, and HLA-DR, reflecting a more immature MC phenotype.^{6,39-41} In line with this, BMMCs from patients with ASM and MCL also displayed abnormally low levels of cytoplasmic tryptase and CPA in association with decreased light scatter features. Interestingly, the marked phenotypic differences observed between these poor-prognosis categories of SM and ISM/cMCAD cases could not be explained on

the basis of a different pattern of *KIT* mutations, because most patients with ASM/MCL also displayed the D816V mutation; however, MCL and ASM typically carry the D816V *KIT* mutation in BMMCs, CD34⁺ cells, and almost all other myeloid cell lineages, in contrast with patients with cMCAD and ISM, in whom the *KIT* mutation is typically restricted to BMMCs.¹⁴ These results suggest that in SM, occurrence of an extended clonal hematopoiesis with multilineage involvement is associated with an earlier blockade of MC maturation among the more aggressive forms of SM; this is further supported by the higher tumor load in the BM and lymphoid tissues and the lower skin involvement typically found among patients with ASM and MCL versus patients with ISM.^{15,42} Further molecular/genetic studies are necessary to elucidate whether such maturation blockade could be a result of the coexistence of additional genetic changes in a D816V⁺ hematopoietic progenitor cell among patients with poor-prognosis SM.

In summary, our results confirm that BMMCs from SM are phenotypically heterogeneous with 3 clearly distinct maturation-associated profiles related to molecular and prognostic subtypes of mastocytosis. More immature immunophenotypic patterns are typically found in ASM and MCL, whereas mature activated (ISM/cMCAD) or resting (WDSM) BMMC phenotypes, dependent on the presence or absence of the D816V *KIT* mutation, are associated with better prognostic subtypes of SM.

Key messages

- Bone marrow MCs from different subtypes of systemic mastocytosis display distinct immunophenotypic profiles.
- The different immunophenotypic profiles of SM are associated with both the *KIT* mutation and the clinical behavior of the disease, with more immature features in aggressive SM and MC leukemia.

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RESULTS

Association between the immunophenotype of clonal BMMCs and other disease features

Patients with ISM with sT levels ≥ 20 $\mu\text{g/L}$ showed higher BMMC counts ($P = .0002$) and lower reactivity for CD25 ($P = .02$) and Fc ϵ RI ($P = .01$); in addition, a significant correlation was found between sT and the mean amount of CD123 ($r^2 = .31$; $P = .04$) per BMMC. Likewise, patients with ISM displaying hepatomegaly and/or splenomegaly had lower expression of Fc ϵ RI ($P = .005$) and CD32 ($P = .04$), together with increased tryptase levels ($P = .009$). Similarly, patients with cMCAD with high sT levels (≥ 20 $\mu\text{g/L}$) showed increased forward light

scatter ($P = .04$), CD59 ($P = .04$), and CD69 ($P = .02$) expression; within these patients, sT levels were also significantly associated with CD64 expression ($r^2 = .55$; $P = .004$).

Among patients with ASM, sT levels showed a significant correlation with the expression of CD63 ($r^2 = .65$; $P = .03$), CD69 ($r^2 = .69$; $P = .01$), and CyB12 ($r^2 = .81$; $P = .006$) per BMMC.

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TABLE E1. Clinical and biological characteristics of adult control subjects and patients with SM grouped according to the diagnostic category of the disease

	Controls (n = 92)	Diagnostic subtype of SM						
		Good-prognosis categories			Poor-prognosis categories			
		WDSM (n = 11)	cMCAD (n = 17)	ISM (n = 69)	ASM (n = 9)	ISM-AHNMD (n = 8)	ASM-AHNMD (n = 6)	MCL (n = 3)
Age (y)	68 ^{c,d,e} (20-89)	34 ^{b,f,g,h} (19-73)	49 ^{b,g,h} (19-73)	43 ^{b,f,g,h} (21-72)	64 ^{c,e} (36-80)	65 ^{c,d,e} (37-74)	72 ^{c,d,e} (60-83)	65 (37-76)
Sex (M/F) ^a	57/35 ^c (62/38)	2/9 ^{b,d,f} (18/82)	13/4 ^c (76/24)	33/36 ^f (49/51)	9/0 ^{c,e} (100)	5/3 (63/37)	3/3 (50/50)	1/2 (33/67)
WBC count (×10 ⁹ /L)	6.0 (3.0-8.6)	5.6 (4.4-7.2)	6.3 (3.5-9.7)	6.1 (3.1-13.2)	5.1 (2.7-13.4)	5.6 (2.2-12.2)	8.0 (4.3-16.8)	4.8 (4.3-9.8)
Skin lesions ^a	NA	11/11 ^l (100)	0/17 ^{c,e,f,g} (0)	68/68 ^{d,m} (100)	3/8 ^{c,d,e} (38)	4/8 ^{c,d,e} (50)	2/6 ^{c,e} (33)	0/3 ^{c,e} (0)
Hepatomegaly ^a	NA	0/11 ^f (0)	1/17 ^f (6)	2/68 ^f (3)	6/8 ^{d,c,e} (75)	3/8 ^e (38)	2/6 (33)	1/3 (33)
Splenomegaly ^a	NA	0/11 ^{f,h,i} (0)	1/17 ^{f,h,i} (6)	4/68 ^{f,h,i} (6)	7/8 ^{c,d,e,g} (88)	2/8 ^f (26)	3/6 ^{c,d,e} (50)	2/3 ^{c,d,e} (67)
Serum tryptase (μg/L)	14.8 ^{d,e,f,g,h} (1.6-23)	11 ^k (4-169)	25 ^{e,l} (15-100)	25 ^{c,m} (3-644)	387 ^{b,c,d,e,g} (100-540)	93 ^{c,n} (8-418)	132 ^{b,c,d,e} (120-469)	713 ^{c,d,e,g} (490-937)
Percentage of BMMCs by flow cytometry	0.02 ^j (0.001-0.5)	0.10 ^{b,i} (0.008-12.0)	0.07 ^l (0.004-1.4)	0.06 ^m (0.0001-1.7)	2.9 ^{b,d,e,g,i} (0.02-15.8)	0.26 ⁿ (0.06-0.9)	4.6 ^{b,d,e,i} (0.02-11.1)	40.0 ^j (26-54)
Mutated <i>KIT</i> ^{a,o,p}	NA	3/10 (30) ^{d,e,f,g,h}	12/14 (86) ^c	57/59 (97) ^{c,i}	9/9 (100) ^c	8/8 (100) ^c	6/6 (100) ^c	2/3 (67) ^c
-Cases analyzed only for purified MC	NA	0/0 (0)	1/1 (100)	7/7 (100)	1/1 (100)	0/0 (0)	1/1 (100)	1/2 (50)
-Cases with all myeloid populations analyzed	NA							
• Only MC	NA	2/10 (20) ^{d,e}	11/13 (85) ^{c,f,h}	35/52 (67) ^{c,f}	0/8 (0) ^{d,e}	3/8 (37.5)	1/5 (20) ^d	0/1 (0)
• MC plus another cell population	NA	1/10 (10)	0/13 (0)	6/52 (12)	1/8 (12.5)	3/8 (37.5)	1/5 (20)	0/1 (0)
• All myeloid BM cells	NA	0/10 (0) ^{f,h}	0/13 (0) ^{f,h}	9/52 (17) ^f	7/8 (87.5) ^{c,d,e}	2/8 (25)	3/5 (60) ^{c,d}	1/1 (100)

y, years; F, female; M, male; NA, not applicable; WBC, white blood cell.

Results expressed as median (range) or as ^anumber of cases/total cases (percentage). Information about the presence or not of skin lesions and hepatosplenomegaly were lacking in 1 ISM and 1 ASM case.Significantly different ($P < .05$, FDR 10%) vs ^bcontrols, ^cWDSM, ^dcMCAD, ^eISM, ^fASM, ^gISM-AHNMD, ^hASM-AHNMD, ⁱMCL, ^jall other groups, and all other groups except: ^kcontrols, ^lWDSM and ISM, ^mWDSM and cMCAD, and ⁿWDSM and ASM-AHNMD.^oNot all patients had mutational analysis, and in some cases only purified MCs (but not other highly purified BM cell fractions) were analyzed.^pAmong patients with *KIT* mutation, 5 had a mutation distinct from D816V (D816Y, I817V, V819Y, and V560G mutations and a V1815-816 insertion were detected each in 1 case).

TABLE E2. Antibodies used for the immunophenotypic analysis of BMDCs

Specificity	Antibody conjugate	Clone	Source
Cytokine receptors	CD25-PE	2A3	BD Biosciences*
	CD117-APC	YB5.B8	BD Biosciences*
	CD123-PE	9F5	BD Biosciences*
Adhesion molecules	CD2-FITC	S5.2	BD Biosciences*
	CD22-PE	S-HCL-1	BD Biosciences*
Complement regulatory proteins	CD59-PE	p282 (H19)	BD Biosciences*
Activation markers	CD63-FITC	CLBGran/12	Immunotech†
	CD69-PE	L78	BD Biosciences*
	CD203c-PE	97A6	Immunotech†
MHC molecules	HLA-I–FITC	B9.12.1	Immunotech†
	HLA-DR–FITC	L234	BD Biosciences*
	HLA-DQ–FITC	TÜ169	BD Biosciences*
Immunoglobulin receptors	CD16-PE	3G8	Immunotech†
	CD32-PE	AT-10	Cytognos‡
	CD64-PE	022CL-3	Immunotech†
Mast cell enzymes	FcεRI-FITC	Polyclonal	Invitrogen§
	Total tryptase–FITC	B12	L. B. Schwartz
	Mature tryptase–FITC	G5	L. B. Schwartz
	Carboxypeptidase A3–FITC	CA2	A. F. Walls¶
Antiapoptotic markers	Anti-Bcl2–FITC	124	Dako#
Other proteins	CD34-FITC	8G12	BD Biosciences*
	CD45-PerCP Cy5.5	2D1	BD Biosciences*

FITC, Fluorescein isothiocyanate; *PE*, phycoerythrin; *APC*, allophycocyanin; *PerCP-Cy5.5*, peridinin chlorophyll protein–cyanin 5.5.

*BD Biosciences (San José, Calif).

†Immunotech (Marseille, France).

‡Cytognos (Cytognos SL, Salamanca, Spain).

§Invitrogen (Invitrogen, Carlsbad, Calif).

||mAb obtained from L. B. Schwartz.^{E1}

¶This mAb was a kind gift from A. F. Walls (Southampton, United Kingdom).

#Dako (Dako, Glostrup, Denmark).

TABLE E3. Immunophenotypic profile of BMMCs from patients with SM grouped according to the diagnostic subtypes of the disease

Phenotypic markers	ISM/cMCAD	WDSM	ASM/MCL
Light scatter	↑	↑	↓/N
CD45	↑	N	N
CD117	N	N	↓
CD123	↑	N	↑
CD25	+	-	+
CD2	+	-	-
CD63	↑	N	N/↑
CD69	↑	N/↑	N/↑
CD203c	↑	N	↓/N
CD22	↑	↑	↑
CD59	↑	N	↑
FcεRI	N/↑	N/↑	↓
CD16	↑	↑	N/↑
CD32	↑	N/↑	↑
CD64	↑	N	↑
HLA-I	N/↑	N	↓/N
HLA-DR	↑	N	N/↑
HLA-DQ	↑	N	N/↑
CyBcl2	N/↑	N/↑	N/↑
CyCPA	N	N/↑	↓/N
CyB12	↓/N	↑	↓
CyG5	↑	N/↑	N
CyB12/CyG5	↓/N	N	↓
Serum tryptase	↑	N	↑
Serum tryptase/CyB12	N/↑	↓/N	↑
Serum tryptase/% of BMMC	↓	↓	↓

CyCPA, Cytoplasmic carboxypeptidase A; N, expression pattern similar to control BMMC; ↑, increased expression versus control BMMC; ↓, decreased expression versus control.

Mast cells from different molecular and prognostic subtypes of systemic mastocytosis display distinct immunophenotypes

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Background: Systemic mastocytosis (SM) is a heterogeneous group of disorders with distinct clinical and biological behavior. Despite this, little is known about the immunophenotypic features of the distinct diagnostic categories of SM.

Objective: To analyze the immunophenotypic characteristics of bone marrow (BM) mast cells (MCs) of different subtypes of SM.

Methods: Bone marrow samples from 123 patients with different subtypes of SM and 92 controls were analyzed for a broad panel of immunophenotypic markers by flow cytometry.

Results: Three clearly different maturation-associated immunophenotypic profiles were found for BMMCs in SM.

These different profiles were associated with both genetic markers of the disease and its clinical behavior. BMMCs from poor-prognosis categories of SM (aggressive SM and MC leukemia) typically showed an immature phenotype with clonal involvement of all myeloid lineages by the D816V stem cell growth factor receptor gene (*KIT*) mutation. In turn, a mature

activated versus resting BMMC immunophenotype was commonly found among patients with good-prognosis subtypes of SM depending on whether they carried (indolent SM and clonal MC activation disorders) or not (well differentiated SM) the D816V *KIT* mutation.

Conclusion: Bone marrow MCs from SM show 3 different maturation-related immunophenotypic profiles that are associated with both the genetic markers of the disease and its clinical behavior. (*J Allergy Clin Immunol* 2010;125:719-26.)

Key words: Mastocytosis, immunophenotype, flow cytometry, *KIT* mutations

Mastocytosis is a heterogeneous group of clonal mast cell (MC) disorders characterized by abnormal proliferation and accumulation of MCs in 1 or multiple tissues.¹ Most frequently, clonally expanded MCs carry the D816V or other activating stem cell growth factor receptor gene (*KIT*) mutations, which translate into morphologic atypia,²⁻⁴ functional transformation,⁵ and an aberrant immunophenotype.⁶ In fact, bone marrow (BM) MCs from systemic mastocytosis (SM) typically exhibit unique immunophenotypic features, and aberrant expression of CD25 and/or CD27 is used as a minor diagnostic criterion for SM.^{1,8} In addition to CD25 and CD2 expression, BMMCs from SM commonly show other aberrancies such as overexpression of the CD63⁹ and CD69¹⁰ activation molecules, CD58—a ligand for the CD2 protein¹¹—CD33,⁷ and several complement-associated molecules—for example, CD11c, CD35, CD59, and CD88.^{6,12} In contrast, expression of kit (CD117),¹¹ the CD71 transferrin receptor, and the CD29 β 1-integrin are abnormally downregulated.⁷

Previous reports suggest that such phenotypic changes of BMMCs in SM could reflect MC activation because of constitutive activating *KIT* mutations.^{6,13} In line with this, recent studies indicate that >90% of all patients with SM carry the D816V *KIT* mutation.¹⁴ However, currently it is well established that SM is not a uniform disease and that it includes several clinicopathological entities and subvariants with different outcomes.^{1,8,15-19} The clinicopathological and prognostic heterogeneity of SM suggests that some patients with SM might carry genetic lesions in addition to the *KIT* mutation (eg, constitutive activation of ras-related protein m-ras^{20,21}), which could contribute to explaining the variable immunophenotypic patterns and interactions of MCs with their microenvironment, similar to what has been demonstrated for other hematologic malignancies.²²⁻²⁴ Despite this, current knowledge about the phenotypic features of different subtypes of SM is rather limited because most phenotypic studies have

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Abbreviations used

ASM:	Aggressive systemic mastocytosis
ASM-AHNMD:	Aggressive systemic mastocytosis associated with a clonal non-mast cell lineage hematopoietic disease
BM:	Bone marrow
cMCAD:	Clonal mast cell activation disorder
CPA:	Carboxypeptidase A
CyB12:	Cytoplasmic total tryptase
CyG5:	Cytoplasmic mature tryptase
FDR:	False discovery rate
ISM:	Indolent systemic mastocytosis
ISM-AHNMD:	Indolent systemic mastocytosis associated with a clonal non-mast cell lineage hematopoietic disease
MC:	Mast cell
MCL:	Mast cell leukemia
NPV:	Negative predictive value
PPV:	Positive predictive value
SM:	Systemic mastocytosis
SM-AHNMD:	Systemic mastocytosis associated with a clonal non-mast cell lineage hematopoietic disease
SSC:	Sideward light scatter
sT:	Serum tryptase
TN:	True negative
TP:	True positive
WDSM:	Well differentiated systemic mastocytosis

either focused on specific subtypes of mastocytosis—for example, indolent SM (ISM)^{7,9,10}—or analyzed a relatively limited number of molecules in relatively restricted cohorts of patients.^{25,26} Furthermore, no relationship between the MC phenotype and the distinct subtypes of SM has been investigated in detail so far. Interestingly, preliminary results^{14,16,17} suggest that the pattern of expression of CD2 and CD25 by BMMCs from well differentiated SM (WDSM)—a recently described variant of SM that frequently lacks D816V *KIT* mutation—could differ from other subtypes of mastocytosis.¹⁴ These findings would further support the existence of a genotypic/phenotypic association among SM.

Here, we analyzed the immunophenotype of BMMCs from a series of 123 patients with SM and compared it among individuals with different subtypes of the disease, as well as with presence or absence of the D816V *KIT* mutation. Our results show that BMMCs from SM are phenotypically heterogeneous with 3 clearly different profiles that are associated with molecular and prognostic subtypes of mastocytosis.

METHODS**Patients, controls, and samples**

A total of 215 BM samples were obtained from adult individuals, including 123 patients (66 men and 57 women; median age, 45 years; range, 19–83 years) consecutively diagnosed with SM²⁷ at the reference centers of the Spanish Network on Mastocytosis (REMA; Mast Cell Unit, Hospital Virgen del Valle, Toledo; and Cytometry Service, Cancer Research Centre, Salamanca, Spain)^{1,8} and 92 normal BM donors, which included 40 normal subjects and 52 patients undergoing BM aspiration for clinical reasons other than mastocytosis (see this article's Table E1 in the Online Repository at www.jacionline.org). In all cases, informed consent was given by each individual before the study, according to the guidelines of the local Ethical Committees.

According to the World Health Organization criteria,^{1,8} patients with SM were classified as follows: ISM, 69 cases; aggressive SM (ASM), 9; MC leukemia (MCL), 3; and SM associated with a clonal non-MC lineage

hematopoietic disease (SM-AHNMD), 14 patients—8 had ISM (ISM-AHNMD), and 6 had ASM (ASM-AHNMD). The other 28 patients with SM corresponded to 2 recently described subvariants of SM: clonal MC activation disorder (cMCAD)^{18,19} (n = 17) and WDSM^{16,17} (n = 11).

Multiparameter flow-cytometry immunophenotypic studies of BMMCs

Multiparameter flow-cytometry immunophenotypic studies were performed on BM aspirate samples collected in Vacutainer tubes containing lithium heparin (Becton/Dickinson—BD-Labware, Franklin Lakes, NJ). All samples were processed within the first 24 hours after they were collected. For sample preparation, a direct immunofluorescence stain-and-then-lyse technique was used, as described elsewhere.⁹ The expression of cytoplasmic markers was evaluated after staining for surface antigens by using the FIX & PERM reagent kit (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Four-color combinations of mAbs were used to stain BM cells with a broad set of reagents (see this article's Table E2 in the Online Repository at www.jacionline.org). For each sample, data acquisition was performed in 2 steps in a FACSCalibur flow cytometer (BD Biosciences) with the CellQUEST software (BD Biosciences) as previously described in detail.⁷ For data analysis, both the INFINICYT (Cytognos SL, Salamanca, Spain) and the Paint-A-Gate PRO (BD Biosciences) software programs were used.

Detection of *KIT* mutation

KIT mutation—D816V or other mutations localized at codons 814 to 819 (exon 17)—was detected on highly purified ($\geq 97\%$ purity) BM cell populations as previously described.^{14,28} In turn, identification of *KIT* mutations at exon 11 was performed on genomic DNA by direct sequencing of the amplified PCR products in both directions, using the dye-deoxy terminator method, in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif) and both the 5'-CCA GAG TGC TCT AAT GAC TG-3' and 5'-AGC CCC TGT TTC ATA CTG AC-3' primers (Isogen Life Sciences, Maarsen, The Netherlands).

Serum tryptase levels

Serum tryptase (sT) levels were determined by using a commercially available standard ELISA technique (Phadia ImmunoCAP Tryptase System; Phadia, Uppsala, Sweden), following the manufacturer's instructions.

Statistical methods

For all continuous variables, median, mean, and SD values, as well as range and the 25th and 75th and the 10th and 90th percentiles, were calculated; for categorical variables, frequencies were reported. Comparisons between groups were performed with either the nonparametric Kruskal-Wallis and Mann-Whitney *U* tests (for continuous variables) or the Pearson χ^2 and Fisher exact tests (for categorical variables); a linear regression model was used to explore the degree of correlation between different variables (SPSS 15.0 software, Chicago, Ill). *P* values $< .05$, with a false discovery rate (FDR) correction for multiple comparisons of $< 10\%$, were considered to be associated with statistical significance.

To classify each case according to the immunophenotypic features of BMMCs, a score was built based on the expression of individual markers. For this purpose, phenotypes were considered aberrant if their divergence from normal/reactive BMMCs was $> \text{mean} \pm 1 \text{ SD}$ and a score of 0 or 1 was given when the MFI of individual markers was $\leq \text{mean} \pm 2 \text{ SD}$ of the mean value obtained for that particular marker depending on whether it was different or coincident with the expression described for the specific diagnostic groups (ISM/cMCAD, WDSM, or ASM/MCL), respectively; a score of 2 and 3 was assigned when the MFI value divergence was $> \text{mean} \pm 2 \text{ SD}$ but $< \text{mean} \pm 3 \text{ SD}$ and when it was $> \text{mean} \pm 3 \text{ SD}$ of the values observed for the corresponding disease category, respectively. Sensitivity was calculated as true positive (TP)/(TP + false negative), specificity as true negative (TN)/(TN + false positive), positive predictive value (PPV) as TP/(TP + false positive),

and negative predictive value (NPV) as $TN/(TN + \text{false negative})$. Receiver operating characteristic curves were used to assess the sensitivity and specificity of immunophenotyping for the diagnosis and classification of SM and its 3 phenotypic subtypes.

RESULTS

Flow-cytometric pattern of BM infiltration by MCs

Patients with SM displayed increased BMMC counts (mean ± 1 SD) versus normal BM ($1.7\% \pm 6.8\%$ vs $0.07\% \pm 0.11\%$; $P < .0001$). ASM, MCL, and SM-AHNMD showed significantly higher BMMC counts than cMCAD and ISM ($P < .05$; Table E1).

Immunophenotypic characteristics of normal/reactive BMMCs

Normal/reactive (control) BMMCs displayed relatively high light scatter values and expressed the CD45, CD117, CD63, CD203c, CD59, FcεRI, CD32, HLA-I, cytoplasmic carboxypeptidase, and cytoplasmic total tryptase (CyB12; Figs 1-3) markers in the absence of reactivity for CD2, CD25, CD123 (Fig 1), and CD34 (not shown). Reactivity for CD64, CD16, HLA-DQ, and HLA-DR (Fig 2) was variable and only detected (partially or dimly expressed) in a restricted number of individuals—5%, 13%, 13%, and 25%, respectively. In turn, CD22 tested positive in half of the controls, with extremely variable—dim to strong—patterns of expression (Fig 2). Mature tryptase (CyG5), CD69, and cytoplasmic b-cell lymphoma 2 protein (CyBcl2) were expressed in the majority of control BM samples—75%, 88%, and 94%, respectively (Figs 1-3). This was associated with relatively high amounts of immature tryptase (high CyB12/CyG5 ratio), low total sT/CyB12 values, and relatively high levels of sT per BMMC (high sT/BMMC ratio; Fig 3).

Overall immunophenotypic features of BMMCs in SM

In comparison with normal BMMCs, clonal BMMCs from patients with SM showed similar patterns of expression of CD117^{hi}, FcεRI⁺, HLA-I⁺, and CD34⁺, except for 1 patient with MCL who expressed CD34 in 28% of the pathological MC. Conversely, they displayed higher light scatter values ($P < .01$), and aberrant expression of CD25^{hi}, CD2, and CD123 in 93%, 72%, and 73% of cases, respectively ($P \leq .0001$; Fig 1). Similarly, expression of CD22, HLA-DR, CD64, CD16, and HLA-DQ, was abnormally increased on BMMCs from most SM: 96%, 85%, 84%, 69%, and 58% of cases, respectively ($P \leq .02$; Fig 2). Reactivity for the CD69, CD63, and CD203c activation markers, FcγRII (CD32), CD45 and the CD59 complement regulatory protein, was also abnormally high in SM ($P < .05$; Figs 1 and 2). In addition, BMMCs from patients with SM showed abnormally low expression of CyB12 with both increased cytoplasmic expression of mature tryptase (CyG5) and higher sT values ($P = .02$), leading to a decreased serum tryptase/BMMC ratio ($P = .01$) and an increased sT/CyB12 ratio (Fig 3).

Aberrant marker expression allowed for a clear discrimination between normal and SM BMMCs, with a sensitivity of 98.1% and a specificity of 100% (PPV, 100%; NPV, 80%).

Differential immunophenotypic profiles of BMMCs from patients with different subtypes of SM

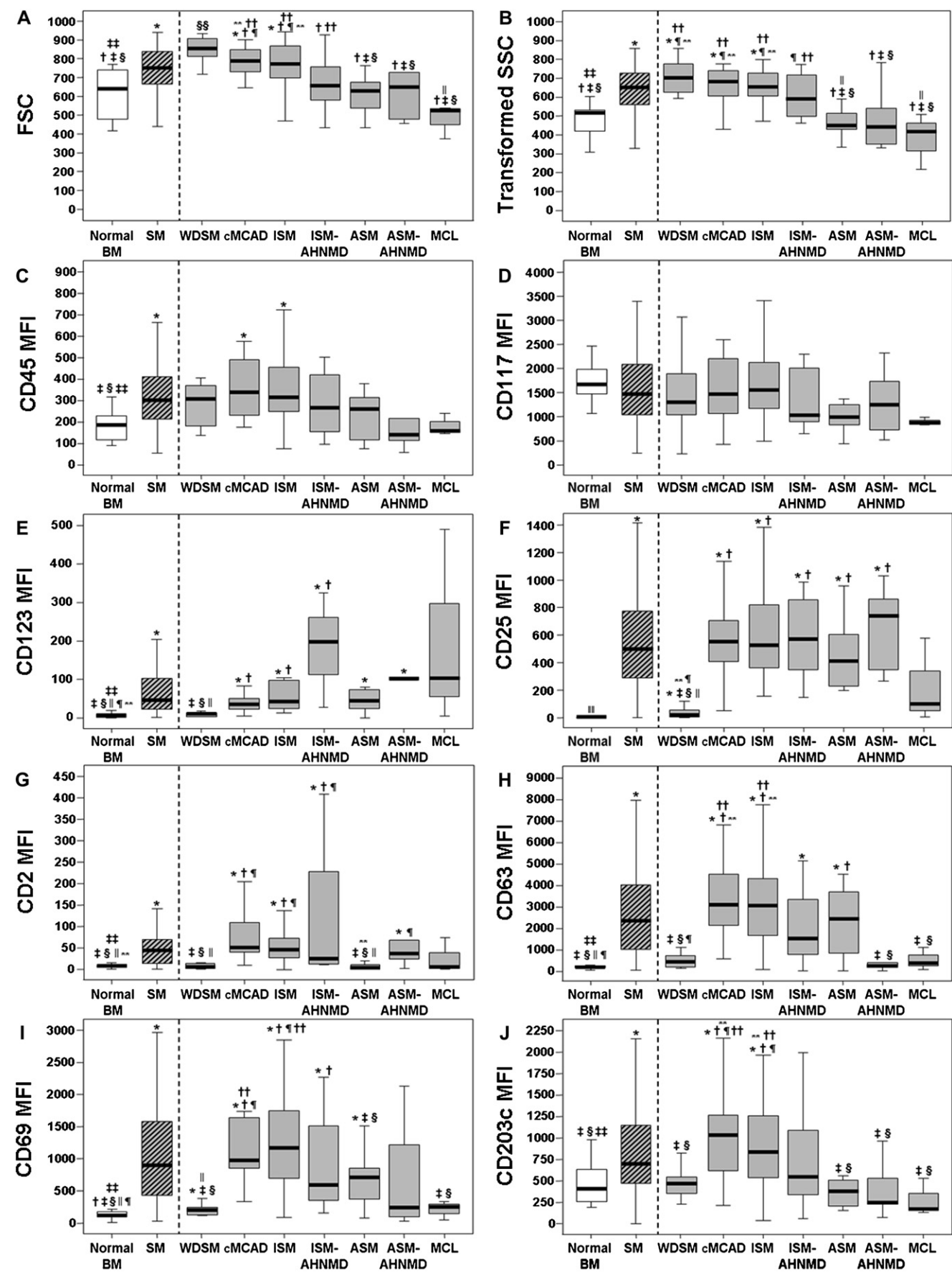
Overall, 3 clearly distinct immunophenotypic profiles were found among SM (see this article's Table E3 in the Online Repository at www.jacionline.org), which corresponded to patients with (1) WDSM, (2) ISM/cMCAD, and (3) both ASM and MCL; SM-AHNMD cases showed a heterogeneous and variable immunophenotype depending on whether the SM component corresponded to ISM or ASM.

Patients with ISM and cMCAD displayed similar immunophenotypic patterns, with aberrantly increased light scatter ($P \leq .003$ vs control BMMCs) and uniform CD25^{hi} expression ($P < .0001$); most cases were also CD2⁺ (88% and 81%, respectively; $P < .0001$; Fig 1). Moreover, BMMCs from cMCAD and ISM showed increased expression of CD16 and CD45 ($P < .005$) and very high reactivity ($P \leq .01$) for CD59, CD63, CD69, CD203c, CD32, CD64, CD123, and HLA-DR (Figs 1 and 2). Although patients with ISM and cMCAD displayed overall increased sT levels ($P < .04$), this was associated with decreased sT levels per BMMC for ISM ($P = .02$; Fig 3); in both groups of patients with SM, higher sT was associated with a decreased CyB12 (total cytoplasmic tryptase).

In contrast with ISM and cMCAD, BMMCs from most patients with WDSM were CD25⁺ and CD2⁺ ($P < .0001$), with only 4 of 11 cases either partially positive for CD25 ($n = 3$) or CD2^{dim}/CD25⁺ ($n = 1$). In addition, BMMCs from WDSM exhibited abnormally increased light scatter ($P \leq .006$ vs control BMMCs; Fig 1), and expression of CD16, CD22 ($P = .003$ vs control BMMCs), CyBcl2 ($P = .006$ vs ISM), cytoplasmic carboxypeptidase, and CyB12 ($P = .003$ vs ISM; Figs 1-3). The greater expression of CyB12 was associated with relatively low sT and a decreased sT/BMMC ratio ($P \leq .008$ vs control subjects, cMCAD, and ISM) but normal total (CyB12)/mature (CyG5) cytoplasmic tryptase levels (Fig 3).

In turn, poor-prognosis SM (ASM and MCL) typically showed an aberrant CD25⁺ (9/9 ASM and 2/3 MCL; $P \leq .001$ vs control BMMCs) but CD2⁺ phenotype with only 1 of 9 patients with ASM and 1 of 3 patients with MCL CD2^{dim} ($P < .0001$ for ASM vs ISM and cMCAD). Likewise, expression of CD63 and CD69 was significantly increased among patients with ASM ($P \leq .02$ vs normal BM) but not patients with MCL (Fig 1). Furthermore, BMMCs from ASM, ASM-AHNMD, and MCL, showed aberrantly low light scatter properties—especially sideward light scatter (SSC; $P \leq .02$ vs good-prognosis SM)—along with decreased CD117 and FcεRI expression ($P \leq .03$ vs normal BM). Other markers, such as CD59 ($P = .02$ for MCL), HLA-DR, CD123, and CD32 ($P < .02$ for ASM), were frequently overexpressed versus controls. Similarly, poor-prognosis variants of SM displayed the highest sT levels ($P \leq .03$ vs good-prognosis SM; Table E1) in association with decreased CyB12 expression leading to an increased sT/CyB12 ratio and decreased sT levels per BMMC (Fig 3).

As described, ISM-AHNMD and ASM-AHNMD displayed similar phenotypic profiles to their ISM and ASM non-associated with a clonal non-mast cell lineage hematopoietic disease counterparts, except for CD117, FcεRI, CyB12, and CD123, whose expression in ISM-AHNMD was closer to that observed among ASM than to ISM (Figs 1-3). Regarding ASM-AHNMD, phenotypic features intermediate between those of ASM and MCL were found for most of the parameters studied, except for CD2



($P = .04$), CD16 ($P = .03$), and CyG5, which showed a higher and more heterogeneous expression in ASM-AHNMD versus ASM (Figs 1-3).

Based on the expression of individual phenotypic markers, prediction of the specific subtype of SM (WDSM vs ISM/cMCAD vs ASM/MCL) could be achieved with a high sensitivity (67%, 86%, and 100%, respectively) and specificity (100%, 86%, and 88%, respectively; PPV, 100%, 94%, and 62%, respectively; NPV, 96%, 71%, and 100%, respectively). Associations between the immunophenotype of clonal BMMCs from the distinct subtypes of SM and the clinical features of the disease (sT levels, presence of hepatomegaly and/or splenomegaly) have also been found (see the Online Results section of this article in the Online Repository at www.jacionline.org).

Frequency and impact of *KIT* mutations on the immunophenotype of BMMCs in SM

Most patients with SM analyzed (97/109; 89%) displayed *KIT* mutations, except for WDSM, for which only 3 of 10 cases carried the *KIT* mutation (the D816V mutation was positive in only 1 of these patients; $P < .05$ vs all SM variants except MCL; Table E1). Interestingly, the frequency of cases carrying *KIT* mutation restricted to the MC compartment was significantly higher in the good-prognosis (ISM and cMCAD) versus poor-prognosis variants (ASM, MCL, or SM-AHNMD; $P < .05$ for ASM vs ISM and cMCAD and, ASM-AHNMD vs cMCAD), which also displayed *KIT* mutation in other nucleated BM myeloid cells (Table E1).

Among those patients who displayed *KIT* mutations, 95% carried the D816V *KIT* mutation whereas other *KIT* mutations were detected in isolated cases (D816Y, I817V, V819Y, and V560G along with a V1815-816 insertion, corresponding to a patient with MCL, a patient with WDSM, and 2 patients with cMCAD, respectively). Overall, no significant phenotypic differences ($P > .05$) were found within each subtype of SM for D816V⁺ BMMCs versus BMMCs showing either other mutations in the activating loop of *KIT* ($n = 4$) or no *KIT* mutational changes at the loci examined ($n = 6$). However, the only patient with cMCAD showing the V560G *KIT* juxtamembrane mutation displayed unique phenotypic features—CD25^{-dim}, CyBcl2^{hi}, CD2⁻, and HLA-I^{dim}—versus other cMCAD cases.

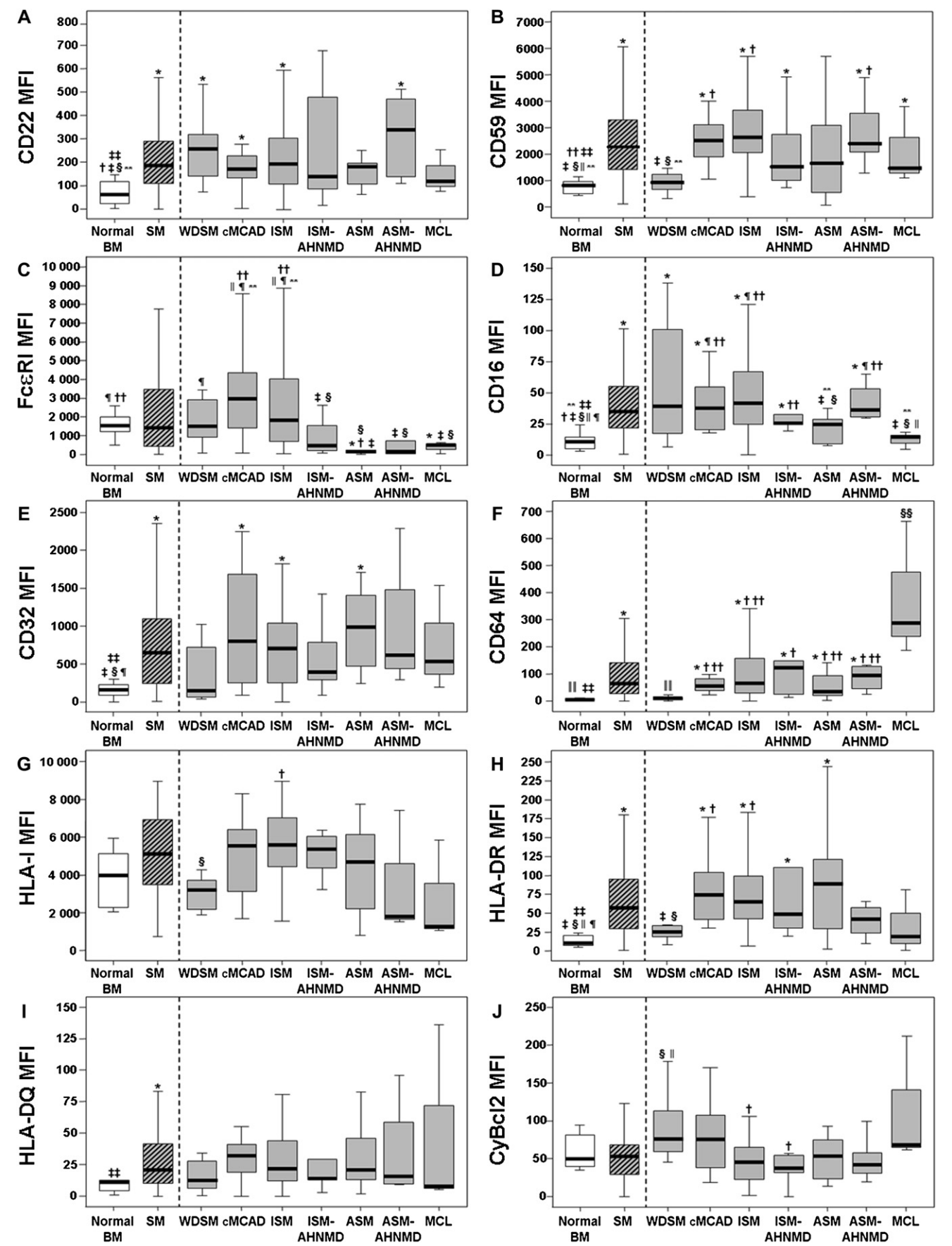
DISCUSSION

Systemic mastocytosis is a clinically and prognostically heterogeneous group of disorders^{1,8,16-19} characterized by the clonal expansion of immunophenotypically aberrant MCs in the patients' BM.^{6,7,9-11} However, little is known about the specific immunophenotypic features of the distinct variants of SM. Here, we analyzed the expression of a broad panel of functional proteins on BMMCs from a large cohort of patients with SM compared with normal/reactive BMMCs. Overall, aberrant phenotypes were identified in all patients with SM, with 3 clearly distinct profiles typically associated with (1) the most common good-prognosis categories of SM (cMCAD and ISM), (2) WDSM, and (3) cases with poor-prognosis subtypes of SM (ASM and MCL).

Currently, aberrant expression of CD25 and/or CD2 on BMMCs represents the only immunophenotypic criterion used in the diagnostic work-up of SM.^{1,8} BMMCs from both cMCAD and ISM showed a typically CD25⁺/CD2⁺ aberrant, mature (eg, FcεRI^{hi}) phenotype associated with overexpression of the CD63, CD69, and CD203c activation markers and the CD64 high-affinity IgG Fc receptor (FcγRI). Interestingly, CD64 is normally absent in resting BMMCs²⁹ but is expressed upon cytokine exposure (INF-γ).^{30,31} Similarly, BMMCs from patients with cMCAD and ISM also showed increased expression of MHC class II molecules (HLA-DR and HLA-DQ) that are typically negative in resting mouse and human MCs but upregulated on activated MCs isolated from tissues infected with pathogens and/or stimulated with cytokines—for example, TNF-α and INF-γ—and LPS.³²⁻³⁴ Altogether, these results suggest that BMMCs from patients with cMCAD and ISM display a phenotypic profile similar to that of activated mature MCs, with aberrant expression of CD2 and CD25. Because virtually every patient within these subgroups of SM carried the D816V *KIT* mutation,¹⁴ which leads to constitutive activation of kit,¹³ this mutation could be responsible for the aberrant activated phenotype of BMMCs in both groups of SM. This hypothesis would be further supported by the absence of phenotypic differences between BMMCs from patients with cMCAD with the D816V *KIT* mutation versus other mutations in the tyrosine kinase loop domain of *KIT*, whereas the only (cMCAD) patient carrying the V560G mutation in the juxtamembrane domain displayed a clearly different immunophenotypic profile.

In contrast with ISM and cMCAD, BMMCs from several other subtypes of SM did not show a CD25⁺/CD2⁺ phenotype. Thus, BMMCs from WDSM were typically CD25⁻/CD2⁻, as previously reported in individual cases¹⁶ and small groups of patients.¹⁷ Furthermore, BMMCs from WDSM also showed normal expression of the CD59, CD203c, and/or CD63 activation markers, which are typically overexpressed by BMMCs from other subgroups of SM.^{6,9,10,12} In fact, BMMCs from WDSM showed a phenotype similar to that of normal resting mature BMMCs, with strong expression of CD117 and FcεRI.^{6,35} In turn, aberrant phenotypes expressed by WDSM were restricted to a few number of cytoplasmic antigens (eg, CyBcl2, carboxypeptidase A [CPA], and tryptase). The increased expression of CyBcl2 (Fig 3) could reflect an altered regulation of apoptosis associated with increased survival of MCs³⁶ in WDSM, as previously suggested for cutaneous mastocytosis.³⁷ In turn, the greater amount of cytoplasmic enzymes (eg, tryptase and CPA) could contribute to the typical hypergranulated morphologic appearance and the abnormally increased SSC of BMMCs in WDSM. Overexpression of cytoplasmic tryptase (CyB12) in association with relatively low sT could reflect an impaired secretion phenotype with a significantly decreased release of tryptase per BMMCs in WDSM versus normal BM. Of note, the overall increased cytoplasmic levels of tryptase—pro and mature α/β-tryptase identified by the B12 mAb—detected in WDSM were associated with a normal total/mature tryptase ratio (CyB12/CyG5). Altogether, these results suggest that spontaneous secretion of protryptase could

FIG 1. Light scatter (forward light scatter (FSC), **A**; sideward light scatter (SSC), **B**) and immunophenotypic characteristics (**C-J**) of BMMCs from adults with different subtypes of SM versus normal/reactive BMMCs, gated as CD117^{hi}/CD45⁺ with intermediate-to-high scatter. *MFI*, Mean fluorescence intensity/BMMC. $P < .05$ and FDR 10% versus *controls, †WDSM, ‡cMCAD, §ISM, ||ISM-AHNMD, ¶ASM, **ASM-AHNMD, ††MCL, ‡‡SM, §§all other groups except SM, and ||||all other groups except SM and WDSM.



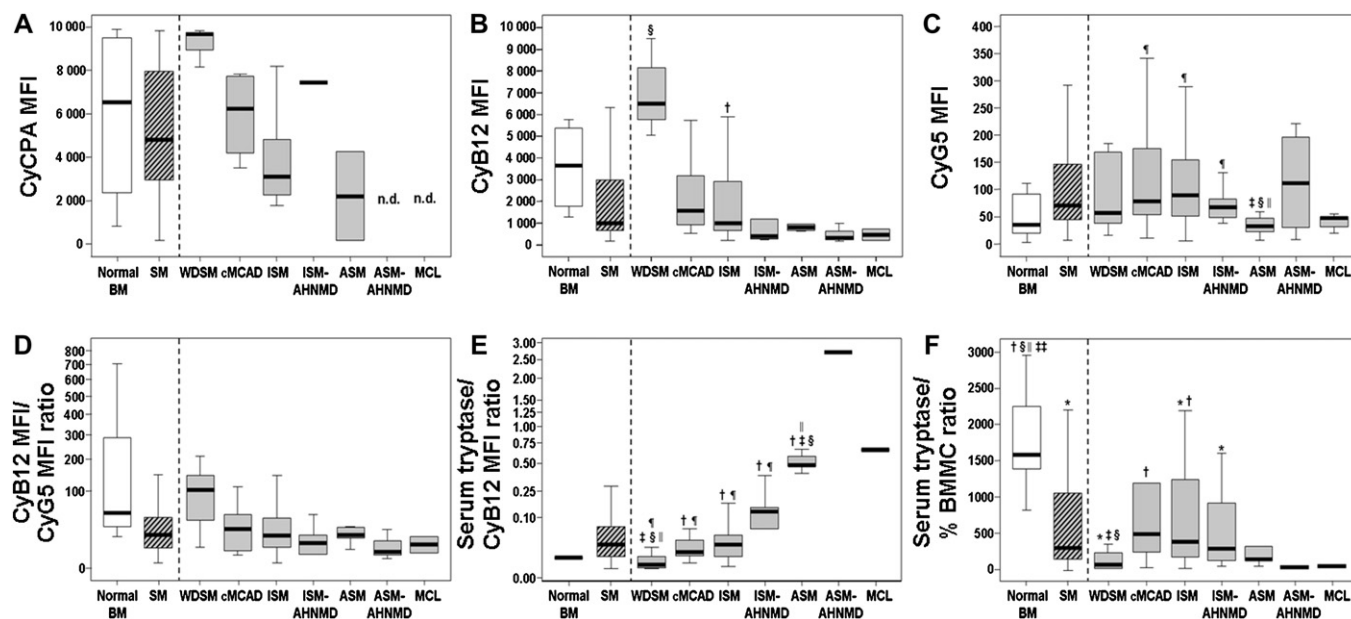


FIG 3. Serum tryptase levels ($\mu\text{g/L}$) and cytoplasmic expression of MC enzymes (mean fluorescence intensity [MFI]) per BMMC (CD117^{hi}/CD45⁺ with intermediate-to-high scatter cells) from patients with SM versus normal/reactive BMMCs. **A-C**, Expression of cytoplasmic carboxypeptidase A (CPA), total (B12) and mature (G5) tryptase per BMMC. **D**, The ratio between the mean amount of total/mature tryptase per BMMC. **E and F**, The ratio between sT and both CyB12 per BMMC and the percentage of BMMCs, respectively. $P < .05$ and FDR 10% versus *controls, †WDSM, ‡cMCAD, §ISM, ||ISM-AHNMD, ¶ASM, and ††ASM. ND, Not done.

be affected in these patients, because protryptase is spontaneously secreted whereas mature tryptase is stored in granules and released in response to MC stimulation.³⁸ Furthermore, because most patients with WDSM did not show *KIT* mutation and those few patients carrying *KIT* mutations displayed a phenotype similar to that of the nonmutated cases, it could be speculated that BMMCs from WDSM may carry additional mutations/genetic changes involving other proteins downstream of kit, which could be responsible for the impaired MC secretion phenotype.

In contrast with other subgroups of SM, the poor-prognosis variants (ASM and MCL) displayed aberrant positivity for CD25, usually in the absence of CD2. This aberrant phenotype was associated with decreased expression of CD117, Fc ϵ RI, and HLA-I and increased positivity for CD123, HLA-DQ, and HLA-DR, reflecting a more immature MC phenotype.^{6,39-41} In line with this, BMMCs from patients with ASM and MCL also displayed abnormally low levels of cytoplasmic tryptase and CPA in association with decreased light scatter features. Interestingly, the marked phenotypic differences observed between these poor-prognosis categories of SM and ISM/cMCAD cases could not be explained on the basis of a different pattern of *KIT* mutations, because most patients with ASM/MCL also displayed the D816V mutation; however, MCL and ASM typically carry the D816V *KIT* mutation in BMMCs, CD34⁺ cells, and almost all other myeloid cell lineages, in contrast with patients with cMCAD and ISM, in whom the *KIT* mutation is typically restricted to BMMCs.¹⁴ These results suggest that in SM, occurrence of an extended clonal hematopoiesis with

multilineage involvement is associated with an earlier blockade of MC maturation among the more aggressive forms of SM; this is further supported by the higher tumor load in the BM and lymphoid tissues and the lower skin involvement typically found among patients with ASM and MCL versus patients with ISM.^{15,42} Further molecular/genetic studies are necessary to elucidate whether such maturation blockade could be a result of the coexistence of additional genetic changes in a D816V⁺ hematopoietic progenitor cell among patients with poor-prognosis SM.

In summary, our results confirm that BMMCs from SM are phenotypically heterogeneous with 3 clearly distinct maturation-associated profiles related to molecular and prognostic subtypes of mastocytosis. More immature immunophenotypic patterns are typically found in ASM and MCL, whereas mature activated (ISM/cMCAD) or resting (WDSM) BMMC phenotypes, dependent on the presence or absence of the D816V *KIT* mutation, are associated with better prognostic subtypes of SM.

Key messages

- Bone marrow MCs from different subtypes of systemic mastocytosis display distinct immunophenotypic profiles.
- The different immunophenotypic profiles of SM are associated with both the *KIT* mutation and the clinical behavior of the disease, with more immature features in aggressive SM and MC leukemia.

FIG 2. Expression of (mean fluorescence intensity [MFI]/BMMC) CD22 (A), CD59 (B), Fc ϵ RI (C), Fc γ R receptors (D-F), HLA molecules (G-I), and CyBcl2 (J) on BMMCs from adults with different subtypes of SM versus normal/reactive BMMCs (CD117^{hi}/CD45⁺ with intermediate-to-high scatter cells). $P < .05$ and FDR 10% versus *controls, †WDSM, ‡cMCAD, §ISM, ||ISM-AHNMD, ¶ASM, **ASM-AHNMD, ††MCL, †††SM, §§all other groups except SM and ISM-AHNMD, and ||||all the other groups except SM and WDSM.

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RESULTS

Association between the immunophenotype of clonal BMMCs and other disease features

Patients with ISM with sT levels ≥ 20 $\mu\text{g/L}$ showed higher BMMC counts ($P = .0002$) and lower reactivity for CD25 ($P = .02$) and Fc ϵ RI ($P = .01$); in addition, a significant correlation was found between sT and the mean amount of CD123 ($r^2 = .31$; $P = .04$) per BMMC. Likewise, patients with ISM displaying hepatomegaly and/or splenomegaly had lower expression of Fc ϵ RI ($P = .005$) and CD32 ($P = .04$), together with increased tryptase levels ($P = .009$). Similarly, patients with cMCAD with high sT levels (≥ 20 $\mu\text{g/L}$) showed increased forward light

scatter ($P = .04$), CD59 ($P = .04$), and CD69 ($P = .02$) expression; within these patients, sT levels were also significantly associated with CD64 expression ($r^2 = .55$; $P = .004$).

Among patients with ASM, sT levels showed a significant correlation with the expression of CD63 ($r^2 = .65$; $P = .03$), CD69 ($r^2 = .69$; $P = .01$), and CyB12 ($r^2 = .81$; $P = .006$) per BMMC.

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TABLE E1. Clinical and biological characteristics of adult control subjects and patients with SM grouped according to the diagnostic category of the disease

	Controls (n = 92)	Diagnostic subtype of SM						
		Good-prognosis categories			Poor-prognosis categories			
		WDSM (n = 11)	cMCAD (n = 17)	ISM (n = 69)	ASM (n = 9)	ISM-AHNMD (n = 8)	ASM-AHNMD (n = 6)	MCL (n = 3)
Age (y)	68 ^{c,d,e} (20-89)	34 ^{b,f,g,h} (19-73)	49 ^{b,g,h} (19-73)	43 ^{b,f,g,h} (21-72)	64 ^{c,e} (36-80)	65 ^{c,d,e} (37-74)	72 ^{c,d,e} (60-83)	65 (37-76)
Sex (M/F) ^a	57/35 ^c (62/38)	2/9 ^{b,d,f} (18/82)	13/4 ^c (76/24)	33/36 ^f (49/51)	9/0 ^{c,e} (100/0)	5/3 (63/37)	3/3 (50/50)	1/2 (33/67)
WBC count (×10 ⁹ /L)	6.0 (3.0-8.6)	5.6 (4.4-7.2)	6.3 (3.5-9.7)	6.1 (3.1-13.2)	5.1 (2.7-13.4)	5.6 (2.2-12.2)	8.0 (4.3-16.8)	4.8 (4.3-9.8)
Skin lesions ^a	NA	11/11 ^l (100)	0/17 ^{c,e,f,g} (0)	68/68 ^{d,m} (100)	3/8 ^{c,d,e} (38)	4/8 ^{c,d,e} (50)	2/6 ^{c,e} (33)	0/3 ^{c,e} (0)
Hepatomegaly ^a	NA	0/11 ^f (0)	1/17 ^f (6)	2/68 ^f (3)	6/8 ^{d,c,e} (75)	3/8 ^e (38)	2/6 (33)	1/3 (33)
Splenomegaly ^a	NA	0/11 ^{f,h,i} (0)	1/17 ^{f,h,i} (6)	4/68 ^{f,h,i} (6)	7/8 ^{c,d,e,g} (88)	2/8 ^f (26)	3/6 ^{c,d,e} (50)	2/3 ^{c,d,e} (67)
Serum tryptase (μg/L)	14.8 ^{d,e,f,g,h} (1.6-23)	11 ^k (4-169)	25 ^{e,l} (15-100)	25 ^{c,m} (3-644)	387 ^{b,c,d,e,g} (100-540)	93 ^{c,n} (8-418)	132 ^{b,c,d,e} (120-469)	713 ^{c,d,e,g} (490-937)
Percentage of BMMCs by flow cytometry	0.02 ^j (0.001-0.5)	0.10 ^{b,i} (0.008-12.0)	0.07 ^l (0.004-1.4)	0.06 ^m (0.0001-1.7)	2.9 ^{b,d,e,g,i} (0.02-15.8)	0.26 ⁿ (0.06-0.9)	4.6 ^{b,d,e,i} (0.02-11.1)	40.0 ^j (26-54)
Mutated <i>KIT</i> ^{a,o,p}	NA	3/10 (30) ^{d,e,f,g,h}	12/14 (86) ^c	57/59 (97) ^{c,i}	9/9 (100) ^c	8/8 (100) ^c	6/6 (100) ^c	2/3 (67) ^c
-Cases analyzed only for purified MC	NA	0/0 (0)	1/1 (100)	7/7 (100)	1/1 (100)	0/0 (0)	1/1 (100)	1/2 (50)
-Cases with all myeloid populations analyzed	NA							
• Only MC	NA	2/10 (20) ^{d,e}	11/13 (85) ^{c,f,h}	35/52 (67) ^{c,f}	0/8 (0) ^{d,e}	3/8 (37.5)	1/5 (20) ^d	0/1 (0)
• MC plus another cell population	NA	1/10 (10)	0/13 (0)	6/52 (12)	1/8 (12.5)	3/8 (37.5)	1/5 (20)	0/1 (0)
• All myeloid BM cells	NA	0/10 (0) ^{f,h}	0/13 (0) ^{f,h}	9/52 (17) ^f	7/8 (87.5) ^{c,d,e}	2/8 (25)	3/5 (60) ^{c,d}	1/1 (100)

y, Years; F, female; M, male; NA, not applicable; WBC, white blood cell.

Results expressed as median (range) or as ^anumber of cases/total cases (percentage). Information about the presence or not of skin lesions and hepatosplenomegaly were lacking in 1 ISM and 1 ASM case.Significantly different ($P < .05$, FDR 10%) vs ^bcontrols, ^cWDSM, ^dcMCAD, ^eISM, ^fASM, ^gISM-AHNMD, ^hASM-AHNMD, ⁱMCL, ^jall other groups, and all other groups except: ^kcontrols, ^lWDSM and ISM, ^mWDSM and cMCAD, and ⁿWDSM and ASM-AHNMD.^oNot all patients had mutational analysis, and in some cases only purified MCs (but not other highly purified BM cell fractions) were analyzed.^pAmong patients with *KIT* mutation, 5 had a mutation distinct from D816V (D816Y, I817V, V819Y, and V560G mutations and a V1815-816 insertion were detected each in 1 case).

TABLE E2. Antibodies used for the immunophenotypic analysis of BMDCs

Specificity	Antibody conjugate	Clone	Source
Cytokine receptors	CD25-PE	2A3	BD Biosciences*
	CD117-APC	YB5.B8	BD Biosciences*
	CD123-PE	9F5	BD Biosciences*
Adhesion molecules	CD2-FITC	S5.2	BD Biosciences*
	CD22-PE	S-HCL-1	BD Biosciences*
Complement regulatory proteins	CD59-PE	p282 (H19)	BD Biosciences*
Activation markers	CD63-FITC	CLBGran/12	Immunotech†
	CD69-PE	L78	BD Biosciences*
	CD203c-PE	97A6	Immunotech†
MHC molecules	HLA-I-FITC	B9.12.1	Immunotech†
	HLA-DR-FITC	L234	BD Biosciences*
	HLA-DQ-FITC	TÜ169	BD Biosciences*
Immunoglobulin receptors	CD16-PE	3G8	Immunotech†
	CD32-PE	AT-10	Cytognos‡
	CD64-PE	022CL-3	Immunotech†
	FcεRI-FITC	Polyclonal	Invitrogen§
Mast cell enzymes	Total tryptase-FITC	B12	L. B. Schwartz
	Mature tryptase-FITC	G5	L. B. Schwartz
	Carboxypeptidase A3-FITC	CA2	A. F. Walls¶
Antiapoptotic markers	Anti-Bcl2-FITC	124	Dako#
Other proteins	CD34-FITC	8G12	BD Biosciences*
	CD45-PerCP Cy5.5	2D1	BD Biosciences*

FITC, Fluorescein isothiocyanate; *PE*, phycoerythrin; *APC*, allophycocyanin; *PerCP-Cy5.5*, peridinin chlorophyll protein–cyanin 5.5.

*BD Biosciences (San José, Calif).

†Immunotech (Marseille, France).

‡Cytognos (Cytognos SL, Salamanca, Spain).

§Invitrogen (Invitrogen, Carlsbad, Calif).

||mAb obtained from L. B. Schwartz.^{E1}

¶This mAb was a kind gift from A. F. Walls (Southampton, United Kingdom).

#Dako (Dako, Glostrup, Denmark).

TABLE E3. Immunophenotypic profile of BMMCs from patients with SM grouped according to the diagnostic subtypes of the disease

Phenotypic markers	ISM/cMCAD	WDSM	ASM/MCL
Light scatter	↑	↑	↓/N
CD45	↑	N	N
CD117	N	N	↓
CD123	↑	N	↑
CD25	+	-	+
CD2	+	-	-
CD63	↑	N	N/↑
CD69	↑	N/↑	N/↑
CD203c	↑	N	↓/N
CD22	↑	↑	↑
CD59	↑	N	↑
FcεRI	N/↑	N/↑	↓
CD16	↑	↑	N/↑
CD32	↑	N/↑	↑
CD64	↑	N	↑
HLA-I	N/↑	N	↓/N
HLA-DR	↑	N	N/↑
HLA-DQ	↑	N	N/↑
CyBcl2	N/↑	N/↑	N/↑
CyCPA	N	N/↑	↓/N
CyB12	↓/N	↑	↓
CyG5	↑	N/↑	N
CyB12/CyG5	↓/N	N	↓
Serum tryptase	↑	N	↑
Serum tryptase/CyB12	N/↑	↓/N	↑
Serum tryptase/% of BMMC	↓	↓	↓

CyCPA, Cytoplasmic carboxypeptidase A; N, expression pattern similar to control BMMC; ↑, increased expression versus control BMMC; ↓, decreased expression versus control.