GENE/PROTEIN EXPRESSION PROFILES OF BONE MARROW MAST CELLS IN SYSTEMIC MASTOCYTOSIS AND THEIR ASSOCIATION WITH THE SUBTYPE OF THE DISEASE

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Gene/protein expression profiles of bone marrow mast cells in systemic mastocytosis and their association with the subtype of the disease

Perfis de expressão génica e proteica de mastócitos de medula óssea em mastocitose sistémica e a sua associação com o subtipo da doença

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Aos meus pais,

Ao meu irmão

Perseverança

Não digas que o trabalho é desperdiçado,
Nem que o esforço falha ou parece, no fundo;
Não digas que aquele ao dever curvado
É um entre os tantos sonhos do mundo.

Pois não é em vão que em golpes seguidos,
Com pressa medida, em fragor crescente,
O mar actua nos rochedos batidos
E invade a praia, ruidosamente.

É certo que enfrentam suas investidas,
Do seu bater forte parecem troçar,
Esmagam com força as vagas erguidas
E em espuma fazem as ondas rasgar.

Mas ele bate e bate com força
Em dias, semanas, em meses e anos,
Até que apareça mossa sobre mossa
Que mostre seus gastos, pacientes ganhos.

E os anos passam, as gerações vão,
E menores se quedam as rochas cavadas;
Mas ele, com lenta e firme precisão,
Baterá na terra suas altas vagas.

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Como duma árvore é o seu crescer,
Trabalha, trabalha sem ser iludido
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E quando o seu fim de todo obtém,
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"Nenhum homem é uma ilha, completo em si próprio;
cada ser humano é uma parte do continente, uma parte de um todo."

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ABBREVIATIONS

2-CdA: Cladribine

AHNMD: Associated clonal hematological non-mast cell lineage disease

Akt: v-akt murine thymoma viral oncogene homolog 1

AML: Acute myeloid leukemia

APC: Antigen presenting cell

ASM: Aggressive systemic mastocytosis

B12: Total tryptase

Bad: BCL2-associated agonist of cell death

Bcl2: B-cell lymphoma 2

Bim: Bcl-2-like protein 11

BM: Bone marrow

BMM: Isolated bone marrow mastocytosis

C/EBPa: CCAAT/enhancer–binding protein a

C5aR: C5a anaphylatoxin chemotactic receptor

CCL: CC-chemokine ligand

cDNA: Complementary deoxyribonucleic acid

c-Kit: Mast/stem cell growth factor receptor Kit

CM: Cutaneous mastocytosis

CMML: Chronic myelomonocytic leukemia

CMP: Common myeloid progenitor

CPA: Carboxypeptidase A3

CR: Complement receptor

CT: Computerized tomography

CXCL: CXC-chemokine ligand

DAG: Diacylglycerol

DNA: Deoxyribonucleic acid

Drp1: Dynamin related protein 1

ds: Double-stranded

ELISA: Enzyme-linked immunosorbent assay

E-NPP3: Ectonucleotide pyrophosphatase/ phosphodiesterase 3

ERK: Extracellular-signal-regulated kinases

ET$_A$: Endothelin receptor type A

F protein: Fusion protein

FACS: Fluorescence-activated cell sorting

FcαR: IgA receptor

FcεRI: High-affinity IgE receptor

FcγRI: High affinity IgG receptor

FcγRII: Intermediate affinity IgG receptor

FcγRIII: Low affinity IgG receptor

FDR: False discovery rate

FGF2: Fibroblast growth factor 2

FITC: Fluorescein isothiocyanate

FOXO3a: Forkhead box, class O3A

FSC: Forward light scatter

Fyn: Tyrosine-protein kinase Fyn

G5: Mature tryptase

GATA-1: Erythroid transcription factor

GATA-2: Endothelial transcription factor GATA-2

GEF: Guanidine nucleotide exchange factor

GEP: Gene expression profile

GI: Gastrointestinal

GIST: Gastrointestinal stromal tumors

GM-CSF: Granulocyte-macrophage colony-stimulating factor

Grb2: Growth factor receptor-bound protein 2

GTP: Guanosine-triphosphate

Hb: Hemoglobin

HES: Hypereosinophilic syndrome

HIV: Human immunodeficiency virus

HMC-1: Human mast cell-1 (cell line)

HPC: Hematopoietic precursor cells

HPCA-1: Hematopoietic progenitor cell antigen CD34

HUMARA: Human androgen receptor allele

ICAM: Intercellular adhesion molecule

ICU: Intensive Care Unit

IFN-α: Interferon alpha

IFN-γ: Interferon gamma

IL: Interleukin

IP3: Inositol-1,4,5-trisphosphate

ISM: Indolent systemic mastocytosis

ISMs-: Indolent systemic mastocytosis without skin lesions

ISMs+: Indolent systemic mastocytosis with skin lesions

ITIM: Immunoreceptor tyrosine-based inhibitory motifs

JAK: Janus kinase

JNK: c-Jun N-terminal kinase

KSHV: Kaposi's sarcoma herpesvirus

LAMP: Lysosomal-associated membrane protein

LCA: Leukocyte common antigen

LCA: Leukocyte common antigen

LDH: Lactate dehydrogenase

LFA-2: Lymphocyte function-associated antigen-2

LIF: Leukemia inhibitory factor

LILR: Leukocyte Ig-like receptor

LPS: Lipopolysaccharide

LTB$_4$: Leukotriene B4

LTC$_4$: Leukotriene C4

Lyn: Tyrosine-protein kinase Lyn

mAb: Monoclonal antibody

MAFA: Mast cell function-associated antigen

MAP: Mitogen-activated protein

MAPK: Mitogen-activated protein kinase

MCET: Mast cell extracellular trap

MCL: Mast cell leukemia

MCP: Mast cell-committed progenitor

MCP-1: Monocyte chemotactic protein-1

MC$_T$: Tryptase positive only mast cell

MC$_{TC}$: Tryptase positive and chymase positive mast cell

MDS: Myelodysplastic syndrome

MFC: Multiparameter flow cytometry

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

MHSC: Multipotential hematopoietic stem cells

MITF: Microphthalmia-associated transcription factor

MPCM: Maculopapular cutaneous mastocytosis

MPN: Myeloproliferative neoplasm

mRNA: Messenger ribonucleic acid

mSCF: Membrane bound stem cell factor

NA: Not applicable

NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF: Nerve growth factor

Notch-2: Neurogenic locus notch homolog protein 2

NR: Not reported

NRAS: Neuroblastoma RAS viral (v-ras) oncogene homolog

NT: Not tested

NTAL: Non-T cell activation linker

PAF: Platelet activation factor

PAMP: Pathogen-associated molecular patterns

PAR: Protease-activated receptors

PCR: Polymerase chain reaction

PDGFR: Platelet-derived growth factor receptor

PE: Phycoerythrin

PEP: Protein expression profile

PFS: Progression-free survival

PGE$_2$: Prostaglandin E$_2$

PGN: Peptidoglycan

Pgp-1: Phagocytic glycoprotein 1

PI3K: Phosphatidylinositol 3-kinase

PIP2: Phosphatidylinositol-4,5-bisphosphate

PIP3: Phosphatidylinositol-3,4,5-trisphosphate

PKC: Protein kinase C

PLCγ: Phospholipase C gamma

PNA: Peptide nucleic acid

PU.1: Transcription factor PU.1

RA: Retinoic acid

Raf-1: RAF proto-oncogene serine/threonine-protein kinase

RAR: Retinoic acid receptor

REMA: Spanish network on mastocytosis (from Spanish *"Red Española de Mastocitosis"*)

RNA: Ribonucleic acid

ROC: Receiver operating characteristic

RSV: Respiratory syncytial virus

RT-PCR: Reverse transcriptase polymerase chain reaction

sBt: Serum baseline tryptase

SCF: Stem cell factor/Kit-ligand

SFK: Src family kinase

SHP-1: Tyrosine-protein phosphatase non-receptor type 6

SHP-2: Tyrosine-protein phosphatase non-receptor type 11

SIGLEC: Sialic acid-binding Ig-like lectin

SLAMF-5: Signaling lymphocytic activation molecule 5

SM: Systemic mastocytosis

SOCS: Suppressors of cytokine signaling

SOS: Son-of-sevenless

Src: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)

ss: Single-stranded

SSC: Sideward light scatter

sSCF: Soluble stem cell factor homodimer

SSM: Smoldering systemic mastocytosis

STAT: Signal Transducers and Activators of Transcription

SWAP-70: Switch-associated protein 70

Tec: Tec protein tyrosine kinase

TET2: Tet methylcytosine dioxygenase 2

TGF-β: Transforming growth factor beta

TK: Tyrosine kinase

TLR: Toll-like receptor

TNF-α: Tumor-necrosis factor alpha

TPO: Thrombopoietin

UCP2: Mitochondrial uncoupling protein 2

UP: Urticaria pigmentosa

uPAR: Urokinase receptor

US: Ultrasound

UVA: Ultraviolet radiation A

VCAM-1: Vascular cell adhesion protein 1

VEGF: Vascular endothelial growth factor

VLA: Very late activation protein

WDSM: Well-differentiated Systemic Mastocytosis

WHO: World Health Organization

ABSTRACT / RESUMO

Abstract

Systemic mastocytosis (SM) comprises a heterogeneous group of disorders characterized by an abnormal accumulation of clonal mast cells in one or more organ systems. As a result of the clinicopathological heterogeneity of the disease, the World Health Organization currently defines several variants of the disease, including forms with a mild (e.g. indolent systemic mastocytosis – ISM) and subtypes with an aggressive behavior (e.g. aggressive systemic mastocytosis – ASM – and mast cell leukemia – MCL) which show a distinct prognosis and different therapeutic requirements. Despite this clinical heterogeneity, in the great majority of SM patients (>90%), except well-differentiated SM (WDSM), the clonal nature of mast cells is demonstrated by the presence of the D816V activating mutation of *KIT*. Therefore the presence of this mutation *per se* does not account for the clinical, histopathological and prognostic variability observed among the distinct subtypes of SM, the molecular and biological basis underlying this heterogeneous behavior remaining largely elusive. In order to address this issue, in the present work we analyzed the protein and gene expression profiles of bone marrow (BM) mast cells from patients with distinct variants of SM, using multiparameter flow cytometry (MFC) and cDNA-oligonucleotide microarrays, in order to gain insight into the biological mechanisms that contribute to explain the heterogeneity of the disease.

In the first part of the work, we evaluated the expression profile of a broad set of proteins in BM mast cells from 123 patients diagnosed with distinct subtypes of SM. Overall, we detected three different maturation-related protein expression profiles (PEP), associated with either the molecular and the prognostic subtypes of the disease. Accordingly, more immature features were detected in the aggressive forms of the disease (ASM and MCL), whereas indolent variants displayed a mature resting (WDSM) or activated (ISM with – ISMs+ - and without skin lesions - ISMs-) immunophenotype,

depending on the absence *vs.* the presence of the D816V activating mutation of *KIT*. Interestingly, the distinct PEP observed between ISM and ASM/MCL patients carrying the same D816V *KIT* mutation seem to be related with the degree of involvement of hematopoiesis by the *KIT* mutation; ASM/MCL patients typically showed multilineage BM involvement in contrast to ISM patients, in whom this mutation was typically restricted to the mast cell compartment. Altogether, these results suggest that the occurrence of an extended clonal hematopoiesis could be associated with an earlier blockade of mast cell maturation among patients with more aggressive forms of the disease.

Recently, it has been described that around 20% of ISMs+ cases also show multilineage BM involvement by the *KIT* mutation, a disease feature which has been reported to be the most powerful independent prognostic factor for progression of ISMs+ to more aggressive disease (e.g. acute myeloid leukemia, myelodysplastic syndrome or ASM). Based on this knowledge, we hypothesized that an association could exist between the PEP of BM mast cells from ISMs+ patients and the degree of involvement of hematopoiesis by the *KIT* D816V mutation. Unsupervised and supervised analysis strategies, performed on distinct groups of patients, confirmed this hypothesis. Accordingly, mast cells from patients with multilineage BM involvement by the D816V *KIT* mutation exhibited more immature features, whereas those cases with the *KIT* mutation restricted to BM mast cells showed a mature activated immunophenotypic profile, regardless of the diagnostic subtype of the disease. Since detection of multilineage *KIT* mutation currently requires a set of techniques which are not easily available in most routine diagnostic laboratories, we further investigated the potential utility of the assessment of the PEP of BM mast cells from ISM patients, as a surrogate marker for the diagnostic screening of multilineage involvement of hematopoiesis by the *KIT* mutation. For this purpose, we developed a score-based class prediction algorithm for the detection of these later cases. Therefore, such cases were efficiently identified by the presence of an increased BM mast cell burden

associated with aberrant expression of CD25 and an immature FcεRI^{lo}, CD45^{lo}, FSC^{lo} and SSC^{lo} phenotype, in the absence of coexisting normal mast cells in the BM. This algorithm exhibited a high efficiency both in a training group of SM patients and in two additional validation sets of patients, regardless of the diagnostic subtype of the disease; moreover, it also showed a significant impact on the progression-free survival of ISMs+ patients, similar to that of the currently used molecular techniques. Altogether, these results suggest that the immunophenotype of BM mast cells could be used as a surrogate marker for multilineage involvement of BM hematopoiesis by the *KIT* mutation, for prognostic stratification of ISMs+ patients.

In the last part of our work we analyzed the gene expression profile (GEP) of highly-purified BM mast cells from SM patients carrying the D816V *KIT* mutation classified according to the diagnostic subtype of SM *vs.* those of normal/reactive BM mast cells. Overall, our results showed that the presence of the D816V *KIT* mutation is associated with a common GEP, which was mainly characterized by up-regulation of genes involved in innate and inflammatory immune responses, including interferon-induced genes involved in the response to viral infections and complement inhibitory molecules. Most interestingly, a distinct GEP was also found among the different subtypes of SM, reflecting an increased lipid metabolism in ISMs- *vs.* increased transcription and protein processing in ISMs+, and deregulation of apoptosis and cell cycle in ASM.

In conclusion, our results show that the clinical and prognostic heterogeneity underlying the distinct variants of SM is associated with distinct protein and gene expression profiles of BM mast cells from these groups of patients, highlighting the potential nature of relevant secondary genetic lesions and/or microenvironmental alterations which may be common to patients classified together in the distinct diagnostic subgroups of SM.

O termo mastocitose sistémica (MS) abrange um conjunto heterogéneo de doenças caracterizadas por uma acumulação anómala de mastócitos clonais num ou mais órgãos ou tecidos. Como consequência da heterogeneidade clínico-patológica da doença, atualmente a Organização Mundial da Saúde (OMS) define várias variantes de MS, incluindo formas com um comportamento indolente (p. ex: MS indolente – MSI) e outras com caráter mais agressivo (p. ex: MS agressiva – MSA – ou leucemia de mastócito – LM), com distinto prognóstico e necessidades terapêuticas. Apesar desta heterogeneidade clínica, na maioria dos casos (>90%) e com a única exceção das MS bem diferenciadas (MSBD), a natureza clonal dos mastócitos é demonstrada pela presença da mutação ativante de *KIT* D816V. No entanto, esta mutação *per se* não explica a variabilidade observada a nível clínico, histopatológico e prognóstico entre os distintos subtipos diagnósticos da doença. Neste sentido, hoje em dia seguimos sem conhecer com precisão as bases moleculares e biológicas do comportamento heterogéneo da doença. No presente trabalho propusemo-nos analisar os perfis de expressão proteica e génica dos mastócitos de medula óssea (MO) de doentes diagnosticados com distintas variantes de MS, aplicando técnicas de citometria de fluxo multiparamétrica (CFM) e *microarrays* de oligonucleótidos de cDNA, com o objectivo de compreender melhor a base biológica desta doença tão heterogénea.

Na primeira parte do estudo, focámos o nosso interesse na avaliação do perfil de expressão de um conjunto amplo de proteínas em mastócitos de MO de 123 doentes com distintos subtipos de MS. De forma geral, observámos três perfis de expressão proteica (PEP) que refletiam o grau de diferenciação das células neoplásicas e que se associavam com o subtipo molecular e prognóstico da doença. De forma mais específica, as formas agressivas da doença (MSA e LM) apresentavam características fenotípicas de mastócito imaturo, em contraposição com as variantes indolentes, nas

quais os mastócitos de MO apresentavam características fenotípicas de célula madura em repouso (MSBD) ou ativada (MSI com – MSIs+ - e sem – MSIs- lesão cutânea), associadas à ausência ou à presença da mutação ativadora de *KIT* D816V, respectivamente. Curiosamente, as diferenças no PEP observadas entre os doentes com MSI e MSA/LM, que partilham a mesma mutação de *KIT*, parecem estar relacionadas com o grau de afetação clonal da hematopoiese nestes doentes; assim, em indivíduos diagnosticados com MSA/LM esta mutação é tipicamente encontrada em várias linhas celulares da MO, em claro contraste com as MSI, nas quais a mutação estava restringida aos mastócitos. Estes resultados sugerem que a ocorrência de uma hematopoiese clonal pode estar associada a um bloqueio precoce da maturação do mastócito nas formas mais agressivas da doença.

Curiosamente, aproximadamente 20% dos doentes com MSIs+ também apresentam a mutação D816V de *KIT* noutras linhas celulares da MO, constituindo estes doentes um grupo de pior prognóstico, com elevado risco de progressão para formas da doença e para doenças mais agressivas (p. ex: leucemia mieloblástica aguda, síndrome mielodisplásico ou MSA). Tendo em conta o PEP mais imaturo previamente observado nas formas mais agressivas da doença, foi colocada a hipótese da possível existência de uma associação significativa entre o PEP dos mastócitos de MO de doentes com MSIs+ e o grau de afetação das diferentes linhas celulares da MO pela mutação D816V de *KIT*. Para testar esta hipótese usámos duas estratégias de análise diferentes (não supervisionada e supervisionada) em grupos distintos de doentes. Em ambos os casos, nos doentes que apresentavam a mutação de *KIT* em várias linhas celulares, os mastócitos mostravam características mais imaturas, por oposição aos casos em que a mutação estava restringida aos mastócitos, e que exibiam características de células maduras ativadas, independentemente do subgrupo diagnóstico. Considerando que a deteção da mutação de *KIT* nas distintas linhas celulares de MO atualmente requer a aplicação de um conjunto de técnicas que não estão facilmente disponíveis nos laboratórios de diagnóstico clínico de rotina,

decidimos averiguar a utilidade do estudo do PEP de mastócitos de MO de doentes com MS, para a triagem diagnóstica de casos com afetação de várias linhas celulares hematopoiéticas pela mutação D816V de *KIT.* Para tal desenvolvemos um sistema de pontuação para a deteção dos referidos casos. Assim, estes eram facilmente identificados pela co-existência de um aumento da carga mastocitária associada à expressão aberrante de CD25 em células com características imaturas (FcεRI^{lo}, CD45^{lo}, FSC^{lo}, SSC^{lo}), na ausência de uma população de mastócitos normais na MO. Este sistema de pontuação mostrou uma alta eficiência na classificação tanto de um grupo de doentes de *training* como de outros dois grupos adicionais de doentes de validação, independentemente do subtipo diagnóstico de MS. Para além disso o citado sistema de pontuação mostrou também um impacto significativo na sobrevida livre de progressão dos doentes, idêntico ao obtido com a deteção do envolvimento de várias linhas celulares pela mutação D816V de *KIT,* usando técnicas moleculares. Estes resultados sugerem que a avaliação do PEP de mastócitos de MO poderia ser usada para a estratificação prognóstica de doentes com MSIs+.

Na última parte do trabalho, estudámos os perfis de expressão génica (PEG) de mastócitos purificados de MO de doentes com diferentes subtipos de MS portadores da mutação D816V de *KIT*, classificados de acordo com o subgrupo diagnóstico da doença. Os resultados obtidos mostraram que a presença da mutação D816V de *KIT* se associa a um PEG comum, caracterizado principalmente pelo incremento de expressão de genes envolvidos na resposta imune inata e inflamatória, incluindo genes induzidos por interferão implicados na resposta a infeções virais, bem como moléculas inibidoras do complemento. Por outro lado, observaram-se diferentes PEG associados às distintas variantes da doença estudadas; estes perfis específicos refletiam um aumento no metabolismo lipídico nas MSIs- *vs.* um incremento a nível da transcrição e processamento de proteínas em MSIs+, e uma alteração da apoptose e do ciclo celular nas MSA.

Em conclusão, os nossos resultados mostram que a heterogeneidade clínica e prognóstica observada nas distintas variantes de MS está associada a perfis de expressão génica e proteica diferentes nos mastócitos da MO destes doentes, evidenciando a possibilidade da coexistência de lesões genéticas e/ou alterações no microambiente medular adicionais, e potencialmente diferentes, nos distintos subtipos diagnóstico da doença.

Chapter 1 |

INTRODUCTION

Mast cells were first described by Paul Ehrlich in 1878, as "granular cells of the connective tissues" - *mästzellen* - that stained reddish-purple with aniline dyes.[1] For many decades these cells have been primarily implicated in the pathogenesis of allergic reactions and certain protective responses to parasites [reviewed in [2]]. However, this concept has evolved and nowadays, mast cells are viewed as highly versatile tissue-resident cells equipped with a unique *armamentarium* of receptor systems and mediators that enable them to play an important role in a large spectrum of biological activities, including regulation of inflammation, host defense and innate immunity [reviewed in [3]].

In parallel, mast cells have also been shown to be involved in a plethora of pathological conditions, including allergy, autoimmune diseases and neoplastic disorders.[4] Among all these conditions, a unique group of mast cell disorders emerges: mastocytosis. Mastocytosis, is mainly characterized by an abnormal accumulation of clonal mast cells in one or more tissues, leading to local and systemic manifestations which are mainly due to an increased mast cell burden and/or release of mast cell mediators.[5] Interestingly, mastocytosis was first reported by Nettlesship & Tay in 1869 (almost a decade prior to the description of the mast cell itself), as a rare skin disease characterized by a symmetrical spread of pigmented maculopapular lesions and an urticaria-like response to scratching; in 1887, Unna showed that such skin lesions contain mast cell aggregates.[6]

Despite mastocytosis was initially described as being associated with dermatological manifestations, currently it is well-known that the disease may involve distinct organs/tissues and a wide panoply of signs and symptoms.[7,8] Consequently, an increasing number of clinically different disease profiles, associated with unique clinico-biological features and therapeutic requirements have been described, leading to the recognition by the current World Health Organization (WHO) classification of mastocytosis of up to nine distinct variants of the disease.[7] However, despite the well

defined clinical, biological and histopathological features of the distinct WHO variants of mastocytosis, the molecular mechanisms and specific biological functions and pathways underlying the heterogeneity of the disease, still remain largely elusive.

1. MAST CELL BIOLOGY

1.1. Mast cell ontogeny and differentiation

For the first 100 years after their discovery by Paul Ehlrich, mast cells were believed to be a component of the connective tissue, derived from undifferentiated mesenchymal cells, and they were thought to function and die within these tissues.[9] However, Kitamura et al. demonstrated in the 70s that mast cells arise from multipotent hematopoietic progenitors in the bone marrow (BM).[10,11] Unlike other hematopoietic cells which complete their differentiation within the BM, mast cells circulate through the vascular system as immature progenitors, before they migrate into peripheral tissues[9] where they complete the final stages of their maturation under the influence of the local microenvironment (e.g. cytokine milieu), which contributes to determine their final morphologic, phenotypic and functional features.[12]

1.1.1- Mast cell-committed progenitor (MCP) cell

Although mast cells have historically been considered to derive from a common myeloid progenitor (CMP), recent studies suggest that these cells could directly originate from multipotential hematopoietic stem cells (MHSCs)[13] or at most, share a common progenitor with basophils, in which the CCAAT/enhancer–binding protein α (C/EBPα) transcription factor expressed in basophil but not in mast cell progenitors, would play a primary role in the fate decision.[14]

Independently of their controversial early ontogeny, human mast cells can be generated from a CD34$^+$ hematopoietic progenitor cell (HPC)[15] in response to stimulation with stem cell factor/Kit-ligand (SCF), a cytokine produced by BM stromal cells.[16] Early reports, using *in vitro* mast cell-differentiation models, revealed that these progenitors circulate as CD34$^+$/CD117$^+$ agranular mononuclear cells,[17] which express the CD133 (prominin 1) stem cell-associated marker,[18] together with myeloid-

associated antigens such as CD13 (aminopeptidase N)[19] and CD33 (siglec-3),[18] in absence of the CD14 monocytic marker (lipopolysaccharide receptor), CD17 (lactosylceramide)[20] and the FcεRI high-affinity IgE receptor.[17] In addition, these precursors have been frequently shown to express CD38 and to often lack HLA-DR expression.[21] More recent *ex vivo* studies, based on the analysis of BM samples from healthy individuals and patients with myelodysplastic syndromes (MDS), phenotypically identified MCP as a minor subpopulation of CD34^{+} HPC, showing a CD117^{hi} HLA-DR[-/int] profile, whose frequency ranges from <0.001% to 0.02% of the CD34+ HPC compartment (mean of <0.001%±0.005% BM cells).[22]

1.1.2.- *Cytokines, growth factors and transcription factors involved in mast cell differentiation*

The differentiation of CD34+ HPC to the mast cell lineage and their maturation to tissue mast cells, are highly regulated processes which depend on multiple cytokines, growth factors and transcription factors; from all cytokines and growth factors involved in mast cell differentiation, SCF (c-Kit ligand) emerges as the most relevant one [reviewed in [23,24]].

1.1.2.1.- *The SCF/Kit signaling pathway*

The crucial role that SCF and its receptor c-Kit (CD117) play in the development and growth of mast cells was first demonstrated by the lack of mast cells in both connective and mucosal tissues of SCF (c-Kit ligand) deficient (WCBF1-Kitl[Sl]/Kitl[Sl-d])[25] or c-Kit deficient mice (WBB6F1-Kit[W]/Kit[Wv]).[10] Further studies revealed a pivotal role for SCF and c-Kit in mast cell proliferation, maturation/differentiation, survival, adhesion and activation [reviewed in [26,27]].

SCF is a glycoprotein encoded at the steel *(Sl)* locus at chromosome 12q22-q24,[28] which is expressed by a wide variety of cells such as stromal cells,

fibroblasts and endothelial cells,[29] and that shows biological activity as a noncovalently associated homodimer.[30] As a result of alternative splicing and proteolytic cleavage, two isoforms of SCF with different effects on survival and proliferation of hematopoietic cells,[29,31] exist. One isorform contains a cleavage site, which allows its proteolysis and release as a soluble homodimer (sSCF), whereas the other remains membrane bound (mSCF).[28] In turn, c-Kit (SCF receptor) is a 145-kD transmembrane glycoprotein encoded at the white spotting (*W*) locus at human chromosome 4q11-q12,[32] which is expressed in a broad array of cell types, including normal maturing and mature mast cells, hematopoietic progenitors, Cajal cells, melanocytes and germ cells [reviewed in [26,29]]. This proto-oncogene is a member of the class III tyrosine kinase family of proteins which also includes the receptor for the platelet-derived growth factor (PDGFR). Like other type III tyrosine kinases, c-Kit is characterized by an extracellular ligand-binding region containing five immunoglobulin-like repeats (from which the first three are involved in the binding of SCF and the fourth may play a role in receptor dimerization, whereas the function of the fifth is still unknown),[33] a transmembrane sequence, an autoinhibitory juxtamembrane domain, and two intracellular tyrosine kinase domains with an ATP-binding pocket and a kinase activation loop (Figure 1A).[34]

Under physiological conditions, activation of c-Kit occurs by non-covalent binding of SCF homodimers to the immunoglobulin loops of the extracellular domain of the receptor; this induces a conformational change leading to the homodimerization of the receptor and subsequent activation of its intrinsic tyrosine kinase activity. The activated receptor then becomes autophosphorylated on key residues, which serve as docking sites for a number of signal transduction molecules, containing Src-homology 2 (SH2) or phosphotyrosine-binding domains (Figures 1B and C)[reviewed in [26,35]]. Such autophosphorylation of c-Kit allows the activation of multiple downstream signaling pathways which have been described to be critical in mediating the SCF/c-Kit signaling for mast cell differentiation; among others, these
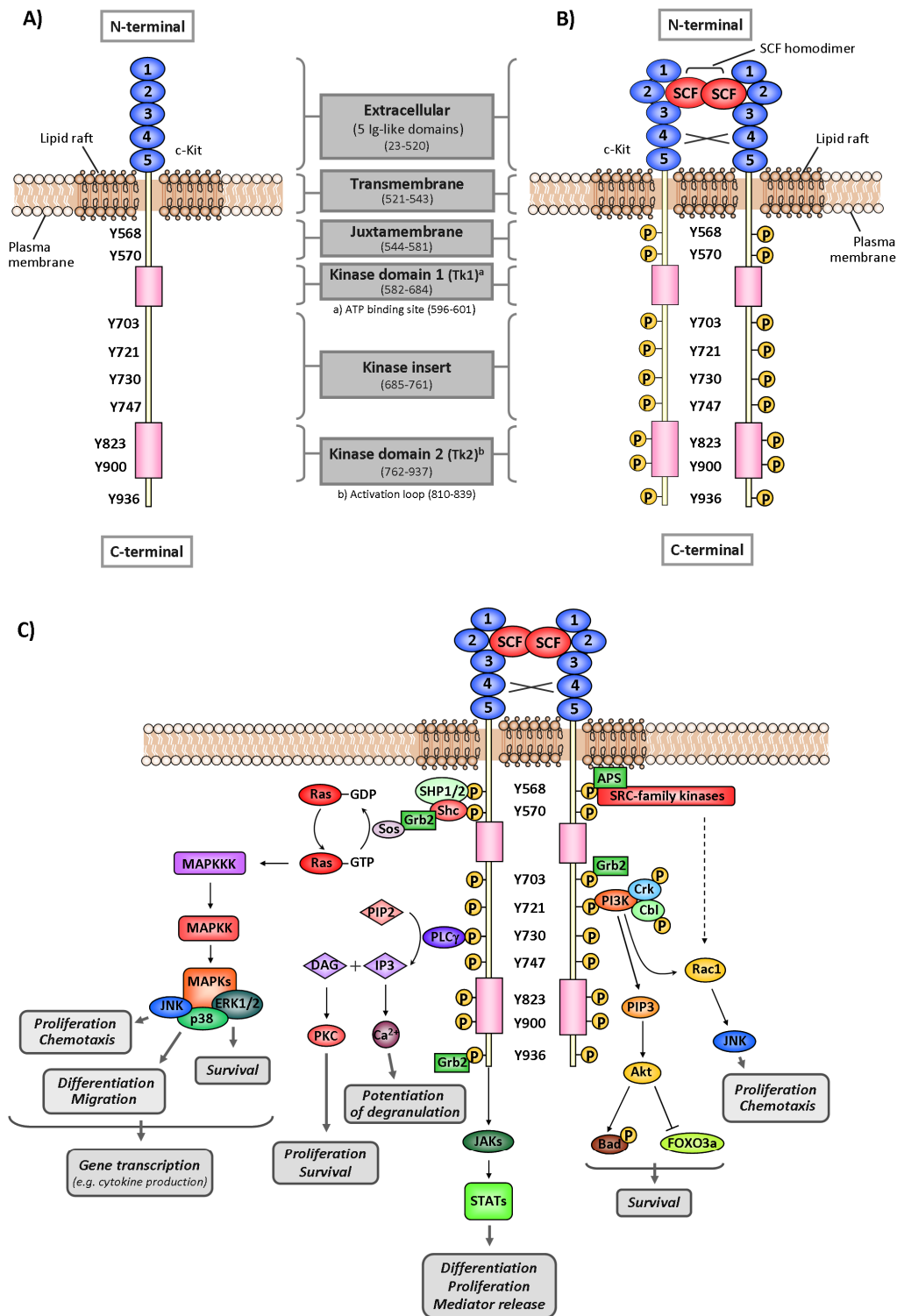
Figure 1. Schematic representation of c-Kit and its tyrosine phosphorylation sites in absence of ligand (A), and upon binding of SCF homodimers and subsequent dimerization of c-Kit (B). Panel C depicts the main downstream signal transduction pathways activated by c-Kit and the major biological functions affected. [Adapted from: Orfao et al.,[26] Lennartsson et al.,[30] Reber et al.[27] and Rönnstrand[35]]

include the Ras/ERK, phosphatidylinositol-3-kinase (PI3-K), Src kinases and the Janus kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) pathways, responsible for eliciting a diverse array of biological responses in both mature mast cells and their precursors (Figure 1C).[26,27,36]

Accordingly, several reports have demonstrated the ability of SCF to activate mitogen-activated protein (MAP) kinase pathways (e.g. the Ras/ERK pathway)[37,38] that play a critical role in cell division and survival [reviewed in [39]]. Following stimulation of mast cells by SCF, the Grb2 (growth factor receptor-bound protein 2) adaptor protein binds c-Kit through both its phosphorylated residues Y703 and Y936[37] and the SHP-2 protein tyrosine phosphatase.[40] Grb2 is present in the cell in a preformed complex with the *son-of-sevenless* (Sos) guanine nucleotide exchange factor, which then interacts with the small G-protein Ras,[41] leading to the activation of Raf-1,[42] and ultimately also of ERK1/2,[42-44] which are known to promote survival by inducing proteasome-dependent degradation of Bim (Bcl-2 interacting modulator of cell death).[45,46] Additionally, activation of c-Kit by SCF also induces activation of other MAP kinases like p38α[44,47] which has been recently suggested to play a critical role in regulating mast cell differentiation and migration,[48] and c-Jun N-terminal kinase (JNK).[47] Thus, through the activation of these protein kinases, which also act as transcription factors, SCF is able to induce the transcription of several genes, including *de novo* synthesis of cytokines (Figure 1C).[49]

SCF/c-Kit-mediated activation of the PI3-kinase downstream pathway has been linked to mitogenesis, differentiation, survival, adhesion, secretion, reorganization of cytoskeletal actin and cell motility.[50-54] Activation of PI3-kinase is a consequence of the direct interaction of PI3-kinase with the phosphorylated Y721 residue of c-Kit, and it leads to activation of Akt.[55] Activated Akt subsequently promotes mast cell survival via inactivation of i) FOXO3a (forkhead box, class O3A) and its transcription target Bim,[56] and ii) the pro-apoptotic factor Bad (through its phosphorylation).[50] In addition, PI3-kinase also mediates SCF-induced proliferation of

mast cells, through the activation of the small guanosine-triphosphate (GTP)-binding protein Rac1/JNK pathway (Figure 1C).[51]

A rapid increase in the activity of Src family kinase (SFK) members (including Src, Tec, Lyn and Fyn) is also observed upon binding of SCF to c-Kit.[57,58] These proteins primarily associate with the phosphorylated Y568 and Y570 residues in the juxtamembrane domain of c-Kit;[59,60] then, they converge to activate Rac1 and JNK, promoting mast cell proliferation and chemotaxis (Figure 1C).[51,61]

Furthermore, the JAK/STAT pathway is also activated following SCF stimulation by rapid and transient phosphorylation of JAK2,[62] which is constitutively associated with c-Kit.[63] Activation of JAK2 leads to phosphorylation of STAT1α, STAT-3 and STAT-5A/B,[63-67] which play an important role in mast cell proliferation and differentiation from a Kit$^+$ progenitor cell (Figure 1C).[68,69] In particular, STAT5 has been shown to contribute to mast cell homeostasis by supporting proliferation, survival and mediator release.[70]

Association of phospholipase Cγ (PLCγ) with residue Y730 of c-Kit has also been previously reported.[71] This enzyme hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2), into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG binds and activates protein kinase C (PKC), whereas IP3 binds to specific receptors present in the endoplasmic reticulum, triggering the release of Ca^{2+} from intracellular stores, which regulates a wide variety of cell functions (Figure 1C).[30] Interestingly, despite sSCF has been described not to induce an increased activity or phosphorylation of PLCγ, studies using mSCF have suggested a role for this protein in SCF-induced proliferation and survival.[30] Moreover, several reports suggest that PLCγ could play a central role in SCF-induced protection against apoptosis induced by irradiation and cytotoxic agents such as danorubicin.[72,73]

Since c-Kit regulates multiple signaling pathways, negative regulation of this receptor occurs at a physiological level through a variety of mechanisms. As other tyrosine kinase receptors, c-Kit is monoubiquitinated upon ligand stimulation, leading

to its internalization and degradation in the lysosomes. Moreover, different molecules have been implicated in down-modulation of c-Kit signaling, including the suppressors of cytokine signaling (SOCS)-1, the SHP-1 protein phosphatase, the CD72 inhibitory receptor and PKC.[35,74,75]

Overall, the biological outcome of the SCF/c-Kit interaction depends on the integrated signals resulting from the activation of multiple interconnected signaling pathways.

1.1.2.2.- *Regulation of SCF-mediated development of mast cells by other cytokines and growth factors*

A remarkable feature of SCF is its capacity to synergize with other hematopoietic growth factors. In fact, a wide array of cytokines and growth factors have been reported to have an important impact in SCF-dependent mast cell growth, differentiation and granule maturation [reviewed in [23,36,76]] (Table 1). Among others, these include molecules with the ability to enhance SCF-dependent mast cell development (e.g. IL-3[77]), either by stimulating SCF-mediated proliferation of mast cells (e.g. IL-5 or IL-9[19,78,79]) or by inhibiting mast cell apoptosis – e.g. nerve growth factor (NGF)-.[80] Furthermore, the impact of the cell microenvironment (e.g. cytokine milieu) on the proliferation and/or differentiation of mast cells in response to SCF varies depending on their degree of differentiation; some growth factors - e.g. thrombopoietin (TPO) - synergistically stimulate mast cell development at early stages,[81] whereas other cytokines (e.g. IL-6) have an impact during the final stages of maturation by inhibiting mast cell growth, decreasing c-Kit expression and increasing cell size, histamine content and the number of chymase positive mast cells.[82] By contrast, other cytokines/growth factors have a negative impact in mast cell development; this is the case of interferon (IFN)-γ, a Th1 cytokine that suppresses SCF-induced differentiation of mast cell progenitors from bone marrow, peripheral blood and cord blood cells.[78,83]

Despite all the above, interpretation of these effects should be made with caution, as most of them derive from *ex vivo* studies, SCF-induced mast cell proliferation or developmental responses are modulated differently depending on the microenvironment (e.g. cytokine milieu)[36] and some contradictory effects are also described in the literature.

Table 1. Effect of distinct molecules (e.g. cytokines and growth factors) on the differentiation and maturation of mast cells. [Modified from: Liu et al.[23] and Okayama et al.[36]]

Type of molecule	Molecule	Receptor	Effect on human mast cells
CYTOKINES AND GROWTH FACTORS	SCF	c-Kit (CD117)	↑ Proliferation of committed progenitors; ↑ Maturation;
	IL-3	IL-3R (CD123)	↑ Proliferation of uncommitted progenitors;
	IL-4	IL-4R (CD124)	Variable depending on MC subtype and cytokine milieu;[a]
	IL-5	IL-5R	Co-factor for proliferation;
	IL6	IL-6R (CD126)	↑ MC growth and ↓ apoptosis;[b] ↓ MC growth and ↑ MC maturation;[b]
	IL-9	IL-9R (CD129)	Co-factor for proliferation;
	IL-10	IL-10R	↓ IgE receptor expression;
	IL-13	IL-13R	↑ MC proliferation;
	NGF	NGFR	↓ Apoptosis in the presence of SCF; ↑ Expression of MC markers;
	IFN-γ	IFNGR1/2	↓ Proliferation;
	TGF-β	TGFR-1/2/3	↓ Proliferation; ↑ Apoptosis;
	GM-CSF	GM-CSFR (CD116)	↓ Proliferation;
	TNF-α	TNF-R1/2 (CD120a/b)	Migration of MC;
	TPO	TPO-R (CD110)	↑ MC development;
OTHER MOLECULES	RA	RAR	↓ Differentiation;

SCF, stem cell factor; IL, interleukin; MC, mast cell; NGF, nerve growth factor; IFN, interferon; TGF, transforming growth factor; GM-CSF, granulocyte macrophage colony stimulating factor; RA, retinoic acid; TNF, tumor necrosis factor; TPO, thrombopoietin.

[a] *IL-4 has been described to inhibit SCF-dependent MC differentiation[84] but also to synergistically enhance the proliferation of human intestinal MC without showing any effect in pulmonary mast cells;[85,86]*
[b] *IL-6 has been widely described to promote mast cell growth and to display an anti-apoptotic activity on blood derived mononuclear cell cultures and CD34+ cells;[19,78,86,87] however, Kinoshita et al. also reported the ability of IL-6 to inhibit MC growth associated with stimulation of maturation.[82]*

1.1.2.3.- <u>Transcription factors involved in the regulation of mast cell</u>
<u>*differentiation*</u>

Current models of hematopoietic development suggest that lineage-specific transcription factors are expressed at low levels in uncommitted precursors.[88] During differentiation, sequential programs of expression of transcription factors are activated, which result in specified sets of dominantly co-expressed transcription factors in committed cells. These lineage-specific sets of co-expressed transcription factors may positively regulate critical gene targets, but they may also function as inhibitors of the activation programs for other cell lineages.[89]

Several transcription factors play a key role in mast cell differentiation but there seems to be no single "master gene" for mast cell development. Indeed, it appears that a combination of the "correct" expression levels of several transcription factors is essential for the activation of the mast cell differentiation program. Among such transcription factors, GATA-1, GATA-2, PU.1 and the microphthalmia-associated transcription factor (MITF) are co-expressed in committed mast cells, and they are required for proper development of the mast cell lineage.[88] GATA-1 is expressed by BM-derived mast cells and mature mast cells,[24] and reduced expression of the GATA-1 gene has been described to lead to an increased apoptosis of MCP and defective differentiation of mature mast cells.[90,91] In turn, GATA-2 and PU.1 are required for early mast cell commitment, since the cooperative expression of these two transcription factors leads to the differentiation of mast cells from BM-derived myeloid progenitors.[92] Furthermore, GATA-2 is essential for the maintenance of early progenitors as well as MCP, since GATA-2 deficient embryonic stem cells are incapable of differentiating into the earliest mast cell progenitors.[93] More recent studies by Taghon et al.[94] suggest that GATA-3 could also play a role in mast cell development, as "forced" expression of GATA-3 in early T-cell precursors (pro-T) can drive development towards mast cell lineage precursors. Overall, the PU.1 and GATA transcription factors seem to be involved in the relatively early stages of development of mast cells as they

would contribute to direct differentiation to the mast cell lineage; by contrast, MITF seems to be involved in a later stage of development and in the migration of mast cell precursors, since it regulates a number of proteases, cell surface receptors, signaling molecules and transporters which are critical for mast cell function. [95]

Other transcription factors which have an impact in mast cell development and differentiation include: i) STAT5, which is a critical downstream regulator of c-Kit signaling;[88] ii) Notch-2, that can induce substantial increase in mast cell numbers, in the appropriate cytokine milieu;[24] and iii) C/EBPα, which seems to play a role in determining the basophil *vs.* mast cell fate.[14]

1.1.3. Normal mast cell maturation

Despite increasing knowledge exists about the factors and mechanisms which influence human mast cell differentiation, the precise functional and phenotypical changes that occur during mastopoiesis remain largely obscure. This is mostly due to the fact that MCP represent a rather infrequent subpopulation in human BM.[22] Therefore, most of our knowledge about the normal patterns of mast cell maturation is based on *in vitro* differentiation studies.[18,96-98] However, this strategy has several drawbacks as it has been described that the immunophenotype, morphology and function of human mast cells is highly variable and that they depend on i) the source of the CD34+ HPC,[99,100] ii) the medium used to culture them[101] and/or iii) the culture conditions.[102] The impact of such factors potentially contributes to explain some of the contradictory results reported in the literature.[18,96] Despite this, consensus exists in the literature about the phenotypic modulation of several molecules involved in various cellular functions, during mast cell maturation. These include early hematopoietic, myeloid and leukocyte lineage markers, cytokines, chemokines and immunoglobulin receptors, as well as activation- and adhesion-associated molecules (Table 2).[18,96,103]

Table 2. Modulation of the immunophenotypic profile of human mast cells during *in vitro* differentiation. [Adapted from Dahl et al,[18] Schernthaner et al,[96] Tedla et al,[97] Yokoi et al,[98] Valent et al[104] and Tachimoto et al[105]]

Functional group of proteins	Surface antigen	CD code	MCP	Cultured immature mast cells (14-28 days)	Cultured mature mast cells (42-80 days)
CYTOKINE, CHEMOKINE AND GROWTH FACTOR RECEPTORS	GM-CSFR	CD116	+	-/+	-
	c-Kit	CD117	+	++	++
	IL-2Rα	CD25	-	-	-
	IL-3Rα	CD123	+	-/+	-
	CXCR1	CD128	-/+	-	-
	CXCR2	CD182	+	+	+
	CXCR4	CD184	+	+	+
ADHESION-MOLECULES	LFA-2	CD2	-	-	-
	Integrin alpha-L	CD11a	+	+dim	+dim
	Integrin alpha-M	CD11b	NR	+dim	+dim
	Integrin alpha-X	CD11c	NR	+dim	+dim
	3-fucosyl-N-acetyl-lactosamine	CD15	NR	+	-/+
	Integrin beta-2	CD18	+	+dim	+dim
	Integrin beta-1	CD29	NR	+	+
	HPCA-1	CD34	+	-/+	-
	Pgp-1	CD44	NR	++	++
	Integrin alpha-2	CD49b	-	-/+	+dim
	Integrin alpha-3	CD49c	-	-/+	+
	Integrin alpha-4	CD49d	+	+dim	+dim
	Integrin alpha-5	CD49e	+	+dim	+dim
	Vitronectin receptor	CD51/CD61	NR	-/+	-/+
	ICAM-1	CD54	NR	-/+	+
	LFA-3	CD58	NR	+	+
IMMUNOGLOBULIN RECEPTORS	FcαR	CD89	-/+	-/+	-/+
	FcεRI	NA	-	-	+
	FcγRI	CD64	-/+	-/+	-/+
ACTIVATION-RELATED MARKERS	LAMP3	CD63	NR	+	+
	Early activation antigen CD69	CD69	NR	-	-
	SLAMF5	CD84	NR	+	+
	E-NPP 3	CD203c	++	++	+
	HLA-DR	NA	-/+	-	-
MAST CELL MEDIATORS AND ENZYMES	Histamine	NA	-	-/+	+
	Chymase	NA	NR	-/+	++
	Tryptase	NA	NR	-/+	++
IMMUNOREGULATORY RECEPTORS	LILRA1	CD85i	NR	-	-
	LILRA2	CD85h	NR	+	-
	LILRB1	CD85j	NR	-/+	-
	LILRB2	CD85d	NR	-/+	-
	LILRB3	CD85a	NR	-/+	-
	LILRB4	CD85k	NR	-/+	-
	LILRB5	CD85c	NR	-	-
	Siglec-2	CD22	-	-	-
	Siglec-3	CD33	+	++	+
	Siglec-5	CD170	+	+	-/+
	Siglec-6	CD327	-	+	+
	Siglec-8	CD329	-	+	+
	Siglec-10	NA	+	-	-

Table 2. *(Continued)*

Functional group of proteins	Surface antigen	CD code	MCP	Cultured immature mast cells (14-28 days)	Cultured mature mast cells (42-80 days)
COMPLEMENT RECEPTORS	CR1	CD35	NR	-	-
	C5aR	CD88	NR	-/+	-/+
OTHER MOLECULES	Aminopeptidase N	CD13	+	+	++
	LCA	CD45	+	+	++

Intensity of expression: (-) negative; (+dim) dim positive; (-/+) heterogeneous from negative to positive; (+) positive; (++) strong positive; MCP, mast cell precursor; NA, not applicable; NR, not reported.

1.1.3.1.- Early hematopoietic, myeloid and leukocyte lineage markers

As previously referred, MPC are known to express a set of early hematopoietic (CD34, CD117 and CD38) and myeloid markers (CD13 and CD33).[15,18,19,21] However, the expression of these molecules varies during mastopoiesis. CD34 expression rapidly decreases in the early stages of maturation, whereas the expression of CD117, CD13 and the CD45 tyrosine phosphatase increases during mast cell differentiation.[18] In turn, CD33 expression increases during the early stages of differentiation while it slightly decreases at the last stages of mast cell maturation.[18,98] By contrast, expression of lymphocyte-related antigens (e.g. CD2, CD3, CD4, CD5, CD10 and CD19) and the CD14 monocytic-related antigen is constantly absent during mast cell differentiation, regardless of the culture conditioning medium used.[96]

1.1.3.2.- Cytokine and chemokine receptors

A significant number of cytokines and growth factors are involved in mast cell development and this translates into varying patterns of expression of cytokine and chemokine receptors during mastopoiesis. Early mast cell progenitors are known to express the IL-3 receptor α-chain (CD123), the granulocyte-macrophage colony-stimulating factor receptor α-chain (GM-CSFRα; CD116), and the IL-6Rα-chain (CD126), in addition to the SCF receptor (CD117) while, except for CD117, these surface antigens, as well as CXCR1, are not detected at later stages of mast cell

maturation.[96,104] In turn, expression of the CXCR3, CCR3 and CCR5 chemokine receptors and both the IL-2Rα-chain (CD25), and gp130 (CD130) cytokine receptors is absent at all stages of mast cell differentiation in *in vitro* culture models.[18,96] Conversely, other cytokine and chemokine receptors such as CXCR4 (CXCL12 receptor), IL-4R and CXCR2 (IL-8 and CXCL1 receptor) are expressed *in vitro* by both immature and mature mast cells.[18,104]

1.1.3.3. Adhesion molecules

Mature mast cells are tissue-resident cells. Therefore expression of adhesion molecules on their surface is required for migration of mast cell progenitors from the bone marrow into peripheral tissues. In detail, previous studies have shown expression of CD29, CD49d, CD49e, CD11a, CD18, CD44, CD54 and CD58 already at the earliest stages of mast cell maturation.[18,96,103] The expression of some of these molecules, such as CD29, CD44 and CD58, remains relatively unchanged during mast cell differentiation, whereas the expression of CD49d, CD49e, CD11a and CD18, decreases.[103] Unlike MCP, more mature mast cells express additional adhesion molecules like CD11b, CD11c, CD51/CD61, CD49b and CD49c.[18,103]

1.1.3.4.-Immunoglobulin (Ig) receptors

Among the set of immunoglobulin (Ig) receptors expressed by mast cells, the most widely known for its functional relevance in these cells is the FcεRI high-affinity IgE receptor. This receptor is absent in MCP[17] but its expression gradually increases during maturation, strong expression levels being typically found in mature mast cells.[18] By contrast, expression of both the FcαR IgA receptor (CD89) and FcγRI IgG high-affinity receptor (CD64) seems to be restricted to a very small subset of the more mature mast cells.[18] Interestingly, expression of both the intermediate (FcγRII;

CD32) and low affinity IgG receptors (FcγRIII; CD16) appears to be dependent on the cytokine milieu.[18]

1.1.3.5. Cell activation markers and complement receptors

Despite MCP typically lack expression of activation-related markers, several of these molecules, like the CD63 lysosomal glycoprotein, the CD84 signaling lymphocytic activation molecule (SLAMF-5) and the CD203c ectoenzyme, are expressed at relatively early stages of mast cell development.[96] Interestingly, CD203c is strongly expressed on the surface of immature mast cells, but its expression is slightly down-regulated thereafter during maturation.[96] Other antigens which are associated with cell activation such as the major histocompatibility complex (MHC) class II molecules (e.g. HLA-DR) are typically absent[96] or show low levels of expression[18] depending on the different culture conditions used, similarly to what has also been described for some complement receptors such as CD35 (CR1) and CD88 (C5aR), whose expression varies depending on the presence or not of IL-4.[96]

1.1.3.6. Other mast cell-associated molecules and Ig-like receptors

During SCF-induced development, MCPs acquire mast cell-specific differentiation enzymes and other markers, such as tryptase, chymase, carboxypeptidase and histamine.[19,96] These molecules appear in an ordered sequential manner during mastopoiesis, e.g. tryptase expression is already detectable at the earliest stages of mast cell development, whereas expression of chymase and histamine have been described to be relatively late events.[16,18,20,106,107] This increase in the cytoplasmic content of mast cell enzymes and mediators is more pronounced at the final stages of mast cell maturation and it is associated with an increase in the size and internal complexity of mature mast cells.[103]

In addition to these mast cell-related antigens, other proteins like the leukocyte Ig-like receptors (LILRs) and the sialic acid-binding Ig-like lectins (Siglecs) are

also expressed throughout mast cell differentiation.[97,98,108] LILRs comprise a family of cell-surface immunoregulatory receptors with both activating and inhibitory members; human cord-blood derived MCP display surface expression of the inhibitory LILRB1 (CD85j), LILRB2 (CD85d), LILRB3 (CD85a) and LILRB4 (CD85k) proteins and the activating LILRA2 (CD85h) receptor, while they do not express the LILRA1 (CD85i) and LILRB5 (CD85c) molecules. In contrast, mature mast cells lack surface expression of all the above LILRs, LILRB5 being detected in the cytoplasmic granules of these cells.[97] Regarding the Siglec receptors, peripheral blood CD34+ progenitors have been shown to constitutively express Siglec-5 and Siglec-10 in addition to CD33 (Siglec-3);[98] as MCP differentiate into mast cells, expression of Siglec-5 and Siglec-10 declines, CD33 expression is maintained, while reactivity for Siglec-6 and Siglec-8 emerges *de novo,* in parallel with accumulation of histamine and other mast cell markers.[98] Interestingly, clear expression of Siglec-2 (CD22) is not detected *in* vitro at any stage of mast cell development.[98]

1.1.4. Immunophenotypic profile of "mature" resting BM mast cells

Despite controversial results have been reported in the literature as regards the maturation-associated immunophenotypic profiles of mast cells, general consensus exists about the immunophenotypic profile of mature, but not terminally differentiated, resting BM mast cells. Such general consensus is probably due to the fact that the assessment of the immunophenotypic features of mature resting BM mast cells is typically performed on *ex vivo* BM samples,[109-112] in the absence of the influence of any additional exogenous factors (e.g. cytokines). Despite this, it should be emphasized that for correct immunophenotypic characterization of mature resting BM mast cells, standardization of the methodology is mandatory.

1.1.4.1. Identification, enumeration and characterization of BM mast cells

Mast cells are mainly tissue-resident cells, which are typically found at very low frequencies in normal human BM (0.008%±0.0082% of all nucleated BM cells).[109] From a morphological point of view, normal BM mast cells display high but heterogeneous light scatter and autofluorescence features,[113] which makes difficult their identification based only on their size and internal complexity. This, together with the lack of specific mast cell-antigens hampers correct identification and characterization of BM mast cells, based on single marker stainings[114] as those used in immunohistochemical techniques. Because of this, in the last decade, multiparameter flow cytometry has become increasingly used for the identification, enumeration and characterization of normal BM mast cells.[112] In this regard, the Spanish Network on Mastocytosis (REMA)[112] has recently provided detailed consensus recommendations for the flow cytometric analysis of BM mast cells, including the need to analyze high numbers of BM cells (between 10^6 to 10^7 BM events) to allow full characterization of CD117^{hi} mast cells, even when these are present at frequencies as low as 10^{-4} - 10^{-5} (Figure 2A and B).[109,111,112,114]

For the specific identification of normal BM mast cells, it is currently well established that strong expression of CD117 (SCF receptor) on cells that coexpress FcεRI and CD203c on their surface membrane, is most useful.[110,115-117] Noteworthy, none of these three proteins is specific of mast cells, because i) CD117 is also detected on a major fraction of hematopoietic precursor cells (HPC),[110,117] CD56$^{+bright}$ NK cells and neoplastic cells from patients diagnosed with several distinct non-mast cell hematologic malignancies (e.g. myelomatous plasma cells and blasts from patients with acute leukemia and myelodysplastic syndromes), as well as on cells derived from other non-hematopoietic tissues,[117] and ii) FcεRI and CD203c are also co-expressed by human basophils in addition to mast cells.[109,118-120]
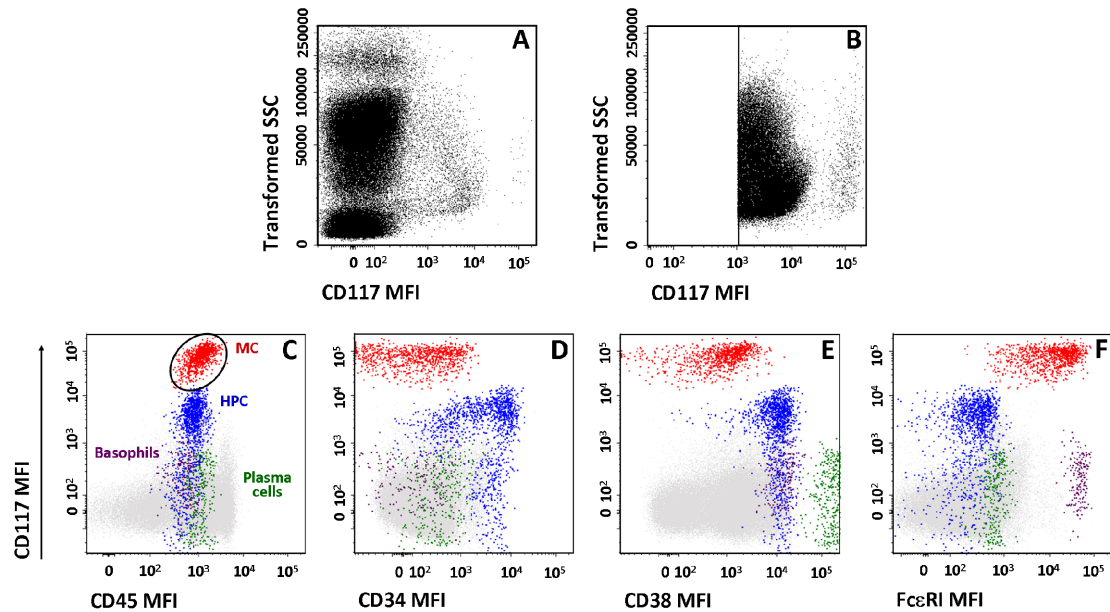
Figure 2. Illustrating example of the gating strategies proposed by the REMA[112] for the immunophenotypic identification and characterization of BM mast cells using multiparameter flow cytometry. Whole BM cell (Panel A) live gate to enrich on CD117^{+} nucleated cells corresponding to 3×10^6 total events (Panel B) from an individual with a BM mast cell load of 0.009% are shown in the upper two panels. Panels C to F show the gating strategy used for the identification of the BM mast cell population (red events) and it phenotypic discrimination from HPC (blue dots), plasma cells (green dots) and basophils (violet dots) coexisting in the same BM sample.

SSC, sideward light scatter; MC, mast cell; HPC, hematopoietic precursor cell; MFI, mean fluorescence intensity (arbitrary units scaled from 0 to 262,000).

Early studies suggested that identification of mast cells could be based on their strong reactivity for CD117 plus FcεRI and CD33, in the absence of expression of CD38, CD34 and CD138, which would allow discrimination between mast cells, CD34^{+} HPC, CD117^{+} plasma cells and CD117^{-/lo} FcεRI^{+} CD33^{+} basophils in BM samples.[109] Nevertheless, simultaneous usage of all six markers for the specific identification of BM mast cells would restrict the possibility for analyzing other proteins of interest (e.g. proteins required to discriminate between normal and pathological mast cells). More recent reports indicate that combined usage of only two markers (CD117 and CD45) would be enough to correctly identify CD117^{high}/CD45^{low} BM mast cells (Figure 2C-F).[112,121] In any case, it should be noted that because of the high and variable autofluorescence levels displayed by highly granular mast cells, determination of

baseline mast cell autofluorescence, using either unstained samples or isotypic controls for the same fluorescence detectors as those used to study the proteins of interest, is mandatory.[112,114,122]

1.1.4.2. Immunophenotypic features of normal and reactive BM mast cells

As mentioned above, mast cells represent an infrequent cell population in normal BM aspirated samples (0.008%±0.0082% of all nucleated cells in adult human BM).[109] Although reactive BM samples and BM samples from patients with non-mast cell related hematological disorders may show slightly increased mean BM mast cell numbers (0.027%±0.17%), these cells typically represent less than 0.1% of the whole BM cellularity.[123] Interestingly, despite the increased frequency of BM mast cells found in reactive samples *vs.* normal BM, few immunophenotypic differences have been described between normal and reactive mast cells.[114] BM mast cells from both healthy subjects and patients with distinct hematologic and non-hematologic non-mast cell related disorders display typical forward light scatter (FSC) and sideward light scatter (SSC) characteristics, similar to those of normal mature mast cells and monocytes,[123] and they also express similar immunophenotypic features as reflected by overlapping levels, to those of normal BM mast cells, of mast cell-related markers (e.g. CD117 and FcεRI) as well as of a wide range of molecules, such as the CD11c, CD29, CD33, CD43, CD44, CD49d, CD49e, CD51 and CD54 adhesion-related proteins, CD9 and CD63 tetraspanins, the CD55 and CD59 complement-receptors, and the CD69 and CD203c activation markers (Table 3).[104,123,124]

Conversely, normal and reactive mast cells do not express the CD34 and CD38 HPC-related markers, the CD1a and HLA-DR MHC-associated molecules, the CD21 and CD88 complement receptors, CD2, CD3, CD5, CD6, CD19 and CD20 lymphoid-associated markers and the CD25 (IL-2Rα) and CD123 (IL-3Rα) cytokine receptors.[110] Interestingly, expression of Siglec-2 (CD22) previously referred as absent on MCP and mature mast cells in *in vitro* differentiation models,[98] is clearly present on BM mast

Table 3. Qualitative and semiquantitative patterns of expression of individual markers on mast cells from normal and reactive bone marrow, as assessed by multiparameter flow cytometry. [Adapted from Escribano et al,[110,113,122] Valent et al,[125] and Nuñez-Lopez et al[124]]

Functional group of proteins	Antigen	CD code	Normal BM (% of positive cases)	Reactive BM (% of positive cases)
CYTOKINE, CHEMOKINE AND GROWTH FACTOR RECEPTORS	IL-2Rα	CD25	-	-
	IL-3Rα	CD123	-	NR
	GM-CSFR	CD116	-	NR
	c-Kit	CD117	+++ (100%)	+++ (100%)
ADHESION-RELATED MOLECULES	LFA-2	CD2	-	-
	Integrin alpha-L	CD11a	-/+ (20%)	ND
	Integrin alpha-M	CD11b	-/+ (50%)	-/+ (50%)
	Integrin alpha-X	CD11c	-/+ (71%)	NR
	3-fucosyl-N-acetyl-lactosamine	CD15	-	-
	Integrin beta-2	CD18	+ (65%)	NR
	Siglec-2	CD22	-/+ (60%)	-/+ (50%)
	Integrin beta-1	CD29	++ (100%)	++ (100%)
	Siglec-3	CD33	++/+++ (100%)	++/+++ (100%)
	HPCA-1	CD34	-	-
	gpIIb/IIIa complex	CD41a	-	NR
	Platelet glycoprotein Ib alpha chain	CD42b	-	NR
	Pgp-1	CD44	++ (100%)	++ (100%)
	Integrin alpha-4	CD49d	+/++ (100%)	NR
	Integrin alpha-5	CD49e	+ (100%)	NR
	Integrin alpha-V	CD51	+ (100%)	+
	ICAM-1	CD54	-/+ (75%)	NR
	Integrin beta-3	CD61	-/+ (66%)	NR
	Carcinoembryonic antigen CGM6	CD66b	-	-
COMPLEMENT-RELATED PROTEINS	CR2	CD21	-	NR
	CR1	CD35	-	+
	Membrane cofactor protein	CD46	+	NR
	Complement decay-accelerating factor	CD55	++ (100%)	++ (100%)
	Membrane attack complex inhibition factor	CD59	++ (100%)	++ (100%)
	C5aR	CD88	-	NR
IMMUNOGLOBULIN RECEPTORS	FcεRI	NA	++/+++ (100%)	++/+++ (100%)
	FcγRIIIB	CD16	-	-
	FcεRII	CD23	-	-
	FcγRI	CD64	-	-
MHC ASSOCIATED MOLECULES	T-cell surface glycoprotein CD1a	CD1a	-	-
	HLA-DR	NA	-	-
TETRASPANINS	CD9 antigen	CD9	+++ (100%)	+++ (100%)
	LAMP3	CD63	++ (100%)	++/+++[a] (100%)
TNF RECEPTOR FAMILY PROTEINS	CD30L receptor	CD30	-	NR
	CD40L receptor	CD40	-/+ (65%)	-/+ (65%)
PROTEASES	Neprilysin	CD10	-	-
	Aminopeptidase N	CD13	-/+ (33%)	NR
	Tryptase	NA	++ (100%)	NR
	Chymase	NA	-/+ [b]	NR

Table 3. *(Continued)*

Functional group of proteins	Antigen	CD code	Normal BM (% of positive cases)	Reactive BM (% of positive cases)
ACTIVATION MARKERS	Early activation antigen CD69	CD69	+ (100%)	+ (100%)
	E-NPP 3	CD203c	+ (100%)	NR
LYMPHOID- AND MYELOID-ASSOCIATED MARKERS	T-cell surface glycoprotein CD3	CD3	-	-
	T-cell surface glycoprotein CD4	CD4	-/+ (60%)	NR
	T-cell surface glycoprotein CD5	CD5	-	-
	T-cell differentiation antigen CD6	CD6	-	NR
	T-cell surface glycoprotein CD8	CD8	-	-
	Monocyte differentiation antigen CD14	CD14	-	-
	B-lymphocyte antigen CD19	CD19	-	-
	B-lymphocyte antigen CD20	CD20	-	-
OTHER MOLECULES	ADP-ribosyl cyclase 1	CD38	-	-
	Leukosialin	CD43	+ (100%)	+ (100%)
	LCA	CD45	++ (100%)	++ (100%)
	Campath	CD52	-	NR
	Ceramide dodecasaccharide	CD65	-	-
	Transferrin receptor protein 1	CD71	+ (100%)	NR
	uPAR	CD87	+	NR
	Syndecan-1	CD138	-	-
	Bcl-2	NA	+ (100%)	+ (100%)
	2D7	NA	-	NR

a) *Expression of CD63 is increased in BM mast cells from myelodysplastic syndromes.*
b) *No available information regarding the frequency of positive cases.*
  NA: not applicable; NR: not reported.

cells from around half the normal and reactive BM samples analyzed.[110] Similarly, expression of the CD11a, CD11b, CD18 and CD61 cell adhesion molecules, as well as aminopeptidase-N (CD13) are only expressed on normal and reactive BM mast cells from a restricted number of cases (Table 3).[126]

Despite all the similarities mentioned above for normal and reactive BM mast cells, two proteins have been reported to show a different pattern of expression in normal *vs.* reactive BM mast cells: i) CD35 (complement receptor 1) is absent in normal BM mast cells but positive in reactive BM and; ii) the CD63 lysosomal glycoprotein, which is constitutively expressed on BM mast cells, is upregulated on mast cells from

myelodysplastic syndrome (MDS) patients *vs.* normal and other reactive BM mast cells.[122,126]

1.1.5. Migration and tissue localization of mast cells

As described above, mast cells derive from BM hematopoietic progenitors that are released into the peripheral blood circulation, at relatively advanced stages of maturation, from where they subsequently migrate into virtually every tissue, to complete their maturation under the control of locally produced cytokines and growth factors. During this process, migration and adhesion are essential for correct tissue localization of mast cells.[29] In fact, migration is an essential function at several stages during mast cell life: i) mast cell progenitors are required to move towards sinusoids in the BM; ii) immature mast cells should cross the sinusoidal endothelium in the BM into the periphery; iii) circulating mast cells are recruited via venules into multiple different tissues, and; iv) they migrate towards their final localization in the tissue.[127] All these processes rely on the ability of mast cells to recognize appropriate chemotactic stimuli and react with appropriate chemotactic responses and adhesion properties. In order to achieve this, MCP and mast cells express numerous surface receptors for various ligands, which act as potent chemoattractants and adhesion molecules.[reviewed in [128]] Among these ligands and their receptors, SCF binding to c-Kit provides critical signals for homing and recruitment of mast cells to various tissues, as both mSCF and sSCF are chemotactic for mast cells and their precursors.[36] Furthermore, SCF promotes cell adhesion by two distinct mechanisms: i) binding of mSCF to c-Kit may directly mediate attachment[129] and, ii) signaling through c-Kit has been shown to upregulate the avidity of mast cell and progenitor cell VLA-4 and VLA-5 β1 integrins, for fibronectin, a specific protein component of the extracellular matrix.[29,127,130,131]

Other relevant mast cell ligands and receptors include adhesion molecules such as the α4β7 integrin, chemokines like CXCR2 (both molecules are involved in the

homing of MCP to the intestine),[132,133] lipid mediators such as leukotriene B4 (LTB$_4$; which is important for the recruitment of progenitors from the microcirculation) and prostaglandin E$_2$ (PGE$_2$; involved in mast cell localization within tissues),[127] antigen binding to IgE anchored to FcεRI, isoforms 1-3 of transforming growth factor (TGF) β, adenosine and complement-associated proteins (e.g. C1q, C3a or C5a), among other molecules [reviewed in [128]]. The relative relevance of each of these molecules is highly dependent on the maturational stage of the cell, since some of them are important for recruiting MCP to tissues (e.g. LTB$_4$ and CCL2), while others mediate mast cell localization within the tissue (e.g. PGE$_2$ or SCF).[78,127] Consequently, the expression of the receptors for most of these ligands is modulated during mast cell development, depending on the phenotypic fate of the cell.

1.1.6. Tissue-associated subsets of mast cells

Despite mast cells share in common many characteristics, distinct subpopulations of mast cells with different morphological, biochemical and functional characteristics, associated with complex phenotypes and tissue-specific microenvironments, can be found.[134,135]

Traditionally, human mast cells have been classified by their cytoplasmic contents on serine proteases as tryptase$^+$ only (MC$_T$) and tryptase$^+$/chymase$^+$ mast cells (MC$_{TC}$), the later also expressing carboxypeptidase A3 and cathepsin G.[136] MC$_T$ cells are predominant in the lung and lamina propria, whereas MC$_{TC}$ prevail in the skin and the intestinal submucosa.[134,136] In addition to their distinct protease content, other significant morphological (e.g. ultrastructure of secretory granules[136]), phenotypical (e.g. expression of the CD88 C5a receptor restricted to MC$_{TC}$[137]) and functional (e.g. T-cell dependence of MC$_T$ but not of MC$_{TC}$[138]) differences are found between these two subtypes of mast cells.

More recently, organ-specific immunophenotypic profiles have been reported for human mast cells (Table 4)[reviewed in [104]]. As an example, expression of the CD32 IgG receptor has been detected on human skin mast cells, but not on lung mast cells;[139] simultaneously, CD88 is clearly expressed on juvenile foreskin mast cells while undetectable on uterine and lung mast cells,[104] and human lung mast cells express significant amounts of ICAM-3 (CD50) whereas their cutaneous counterparts only express undetectable to low amounts of this molecule.[104,139]

Table 4. Immunophenotypic characteristics of human mast cells from distinct tissues.
[Adapted from Valent et al,[104] Krauth et al,[140] Escribano et al[113] and Fureder et al[141,142]]

CD code	Antigen	Tissue of mast cell origin								
		BM	Lung	Foreskin	Uterus	Tonsils	Kidney	Heart	Synovial fluid	GI tract
CD2	LFA-2	-	-	-	-	NT	-	-	-	-
CD9	CD9 antigen	+	+	+	+	+	+	+	+	NT
CD25	IL-2Rα	-	-	-	NT	NT	-	NT	-	NT
CD29	Integrin beta-1	+	+	+	+	NT	NT	+	NT	+
CD32	FcγRII	NT	-	+	NT	NT	NT	NT	NT	-
CD34	HPCA-1	-	-	-	-	NT	-	NT	NT	-
CD35	CR1	-	-	-	-	-	-	-	-	-
CD44	Pgp-1	+	+	+	+	+	+	+	+	+
CD45	LCA	+	+	+	+	+	+	+	+	+
CD50	ICAM-3	NT	+	-/+[a)]	NT	NT	NT	NT	+	NT
CD51	Integrin alpha-V	+	+	+	+	NT	+	NT	NT	NT
CD54	ICAM-1	-/+	+	+	+	NT	NT	+	NT	+
CD61	Integrin beta-3	+	+	+	+	NT	+	+	+/-	NT
CD63	LAMP3	+	+	+	+	+	+	NT	NT	+
CD88	C5aR	-	-	+	-	-	-	-/+[a)]	+/-[b)]	-
CD117	c-Kit	+	+	+	+	+	+	+	+	+
NA	FcεRI	+	+	+	+	+	+	+	+	NT

a) Low or undetectable expression on mast cells.
b) Synovial mast cells in patients with rheumatoid arthritis express CD88, whereas synovial mast cells in patients with osteoarthritis are CD88-negative.
BM, bone marrow; GI, gastrointestinal tract; NA, not applicable; NT, not tested.

1.2. Mast cell activation

Mature mast cells are long-living tissue-resident cells[143] strategically located at the host's interfaces with the environment where they function as sentinels in host

defense by responding to a wide range of "signals" (e.g. environmental antigens, allergens, invading pathogens or toxins) in a rapid and selective way [reviewed in [4]]. For this purpose, they are equipped with a large repertoire of receptors that upon interaction with pathogens or toxins, induce the release of a wide variety of biologically active products and may even lead to *in situ* proliferation of terminally differentiated mast cells (Table 5) [reviewed in [143,144]]. Specific responses to individual stimuli depend on the particular mechanism leading to mast cell activation, the type of the signal, its intensity and duration, as well as the cytokine milieu.[145,146] Altogether, the ability of mast cells to modulate their response to distinct stimuli confers them an enormous functional plasticity.

1.2.1. Mast cell receptor systems and activators involved in host defense

The most widely studied mechanism of mast cell activation is that triggered by cross-linking of the FcεRI high-affinity IgE receptor, leading to degranulation and secretion of newly synthesized mast cell mediators.[4] In addition to FcεRI, mature mast cells constitutively express the intermediate-affinity FcγRII receptor (CD32),[147] and they may also upregulate the expression of other IgG receptors after exposure to cytokines (e.g. expression of the CD64 high-affinity FcγRI IgG receptor is up-regulated upon exposure to IFN-γ).[148] Therefore, these cells can bind both IgE and IgG, and become sensitized to antigens previously encountered by the host.[143]

Despite all the above, the mechanisms and effects of mast cell activation through crosslinking of IgE and IgG to their respective receptors on the mast cell's surface membrane, are significantly different. FcεRI is activated by both antigen-complexed IgE and monomeric IgE,[149-151] whereas Fcγ receptors only associate with antigen-complexed IgG.[4] However, this later class of receptors may lead to either positive or negative regulation of mast cell activation, as IgG linkage to FcγRI (CD64) or

Table 5. Main mast cell receptor systems involved in the host defense against allergens, pathogens, toxins and other stimuli. [Modified from Marshall et al.[147]]

Receptor class	Type of receptor	Examples of ligands	Major biological effect
DIRECT RECEPTORS FOR PAHTOGEN PRODUCTS			
TOLL-LIKE RECEPTORS (TLR)	**TLR1**	Lipopeptide	Eicosanoid and cytokine production and release
	TLR2	PGN, zymosan and some types of LPS	
	TLR3	dsRNA	
	TLR4	LPS and F protein from RSV	
	TLR6	PGN and zymosan	
	TLR7	ssRNA (viral)	
	TLR9	Bacterial DNA and CpG-containg DNA	
MANNOSYLATED RECEPTORS	**CD48**	FimH	Degranulation Cytokine production
INDIRECT RECEPTORS FOR PRODUCTS OF IMMUNE RESPONSES TO PATHOGENS			
Ig RECEPTORS	**FcεRI**	IgE	Degranulation Eicosanoid, cytokine and chemokine release
	FcγRI, FcγRII, FcγRIII	IgG	
COMPLEMENT RECEPTORS	**CR2, CR4 and CR5**	Complement components	Degranulation Production of chemokines ↑ FcγRI-dependent degranulation
	C5aR and C3aR	Complement split products	
CYTOKINE RECEPTORS	**IL-4R**	IL-4	FcεRI-dependent release of histamine, LTC4 and IL-5
	IL5-R	IL-5	FcεRI-dependent release of cytokines
	IL-10R	IL-10	↓ FcεRI-dependent release of cytokines ↓Decreased FcεRI expression
	c-Kit	SCF	Cytokine and chemokine release ↑ Growth, differentiation and adhesion FcεRI-dependent degranulation and cytokine production
	IL-1R, IL-12R, IFN-γR	IL-1, IL-12, IFN-γ	Immunoregulation
CHEMOKINE RECEPTORS	**CCR3**	CCL11	FcεRI-dependent secretion of IL-13
	CCR5, CXCR4	Chemoattractants (chemokines)	Mast cell migration
OTHERS MOLECULES	**Neurotensin receptor**	Neurotensin	Degranulation
	NK1-receptor	Substance P	Degranulation
	ET$_A$	Endothelin-1	Degranulation and eicosanoid release

LPS, lipopolysaccharide; PGN, peptidoglycan; ds; double-stranded; ss; single-stranded; F protein, fusion protein; RSV, respiratory syncytial virus; ET$_A$, Endothelin receptor type A.

FcγRIII (CD16) causes activation of mast cells (through binding of pathogen specific antibodies complexed to antigen and binding of nonspecific antibodies complexed to immunoglobulin-binding proteins, e.g. superantigens produced by pathogens), whereas ligation of FcγRIIb inhibits mast cell functions, including mast cell activation

induced by FcεRI aggregation and mast cell proliferation induced by c-Kit mediated signaling.[4,152,153]

Mast cells are also able to directly interact with several pathogens through the binding of Toll-like receptors (TLRs) to conserved pathogen-associated molecular patterns (PAMPs).[154] Accordingly, human mast cells express TLR1-4, TLR-6, TLR-7 and TLR-9,[155] which provide mast cells with the ability to respond to a wide variety of bacterial - e.g. lipopolysaccharide (LPS) and peptidoglycan (PGN) - and viral (e.g. double stranded RNA) components, leading to the production of multiple mast cell mediators, such as TNF-α, GM-CSF, IL-1β, IL-5, IL-13 and leukotriene C4 (LTC$_4$), usually in the absence of mast cell degranulation.[147,156,157] Similarly, interaction between the FimH fimbrial protein on gram negative bacteria and CD48 on human mast cells, triggers mast cell degranulation, bacterial internalization and cytokine (e.g. TNF-α) production.[135,158]

Many infections and other forms of tissue injury induce the activation of complement. Complement activation ultimately leads to mast cell activation. Mast cells have long been recognized to interact with the complement system through receptors specific for several complement products such as complement receptor (CR) 3 (also known as CD11b-CD18), CR4 (CD11c-CD18) and C3aR/C5aR (CD88)[147,159] As previously mentioned, expression of these complement receptors varies depending on the differentiation stage of mast cells and the cytokine milieu of their environment.[142]

Several specific growth factors and cytokines that are involved in mast cell differentiation from MCP, also play an important functional role in mast cell activation and the release of mast cell mediators. Accordingly, SCF-induced activation of c-Kit has been shown to regulate mediator release by human mast cells, either through the enhancement of IgE-dependent mediator release or by directly inducing mediator release by mast cells kept in an SCF-deprived milieu.[160,161] In addition, SCF may also negatively regulate IgE-dependent mast cell activation, as reflected by a marked attenuation of FcεRI-mediated degranulation and cytokine production as a

consequence of ineffective cytoskeletal reorganization found in mouse BM-derived mast cells chronically exposed to SCF.[162] In contrast to SCF, IL-4 does not affect mast cells by itself, but it synergizes with SCF on inducing IgE-dependent release of mediators.[85,161] Furthermore, priming of mast cells with IL-4 has been shown to increase the expression of leukotriene C4 synthase[163] and enhance the ability of intestinal mast cells to produce Th2-type cytokines,[164] in the context of FcɛRI-mediated mast cell activation. Interestingly, other cytokines such as IL-23 and IL-1β have been recently suggested to induce mast cell degranulation and lead to the production of mast cell extracellular traps in human skin, which would result in the release of IL-17 and other mast cell mediators, in a psoriasis model.[165]

Other mast cell activators include several endogenous host peptides such as neurotensin, substance P and endothelin-1,[143,166] chemical agents and physical stimuli.[167]

1.2.2. Mast cell mediators

Activation of mast cells by distinct stimuli induces both mast cell degranulation and *de novo* synthesis of mast cell mediators (Table 6). Degranulation involves rapid (within seconds to minutes) release of pre-packed, insoluble mediators into the surrounding tissue, a strategy that gives mast cell-derived products a transient advantage over those produced by other immune surveillance cells.[143] Mast cell degranulation may occur by two distinct mechanisms: i) classical anaphylactic degranulation, in which the entire contents of each granule is released by exocytosis, and; ii) piecemeal degranulation, consisting of partial mast cell degranulation due to slow and progressive release of the contents of the granules.[4,168] Each mast cell can undergo multiple rounds of degranulation and regranulation, which allows for the perpetuation of mast cell-mediated responses.[169]

Table 6. Major subtypes of mediators released by mast cells and their most relevant biological effects. [Modified from Marshall et al.[147]]

Mediators	Major biological effects
GRANULE ASSOCIATED MEDIATORS	
Histamine and serotonin	↑ Vascular diameter and permeability
Heparin and/or chondroitin sulphate peptidoglycans	Enhance the effects of chemokines and/or cytokines function; ↑ Angiogenesis
Tryptase, chymase, carboxypeptidase and other proteases	Tissue remodeling Recruitment of effector cells
TNF, VEGF and FGF2	Recruitment of effector cells ↑ Angiogenesis
LIPID-DERIVED	
LTC4, LTB4, PGD2, PGE2	Recruitment of effector cells Regulation of immune responses ↑ Angiogenesis, edema and bronchoconstriction
Platelet activating factor (PAF)	Activation of effector cells ↑ Angiogenesis Induction of physiological inflammation
CYTOKINES	
TNF, IL1-α, IL-1β, IL-6, IL-18, GM-CSF, LIF, IFN-α, IFN-β	Induction of inflammation
IL-3, IL-4, IL-5, IL-9, IL-13, IL-15, IL-16	T helper 2-type cytokines
IL-12, IFN-γ	T helper 1-type cytokines
IL-10, TGF-β, VEGF	Regulation of inflammation and angiogenesis
CHEMOKINES	
CCL2, CCL3, CCL4, CCL5, CCL11, CCL20	Recruitment of effector cells, including dendritic cells Regulation of immune responses
CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, CXCL11	Recruitment of effector cells Regulation of immune responses
OTHER MOLECULES	
Nitric oxide and superoxide radicals	Bactericial effects
Antimicrobial peptides	Bactericial effects

CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; FGF2, fibroblast growth factor 2; GM-CSF, Granulocyte/macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; LTB4, leukotriene B4; LTC4, leukotriene C4; PGD2, prostaglandin D2; PGE2, prostaglandin E2; TGF-β, transforming growth factor-β; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor.

Mast cell mediators packed in cytoplasmic granules mainly consist of proteoglycans, proteases (e.g. tryptase, chymase and carboxypeptidase A), histamine and cytokines (e.g. TNF-α). Proteoglycans, such as heparin and chondroitin sulfates, are abundant components of mast cell granules and due to their negative charge, they form complexes with histamine and proteases, that stabilize these later mediators.[170] Following exocytosis of mast cell granules, some mediators (e.g. histamine) become immediately soluble, whereas most of the exocytosed nanoparticules maintain their structure through tight interactions with (negatively charged) proteoglycans. Therefore,

granule proteins, can remain associated for longer periods as insoluble particles which are slowly released; in addition, these particles can also travel, through the lymphatic vessels, from the site of release (e.g. infection) to the draining lymph nodes, suggesting that inflammatory mediators can be delivered at relatively long distances through proteoglycan-containing mast cell mediator complexes.[143,171]

Neutral proteases (tryptase, chymase, cathepsin G and carboxypeptidase A – CPA-) constitute the majority of the protein contents of mast cell granules. These proteins play an important role in promoting host defense through their ability to recruit other immune cells[172,173] and the activation of protease-activated receptors (PAR).[4] In addition, they are also involved in tissue remodeling through their direct effects on the extracellular matrix and matrix metalloproteinases.[4,174] Furthermore, mast cell proteases may have an important role in limiting the harmful effects of toxic host-derived products of inflammation, such as neurotensin and endothelin.[166,175]

Histamine is a vasoactive amine, with a wide range of effects mediated through four (H1, H2, H3 and H4) receptors.[146,176] H1 receptors control the tone and permeability of the vascular bed and the intestinal and bronchial smooth muscle, as well as the production of mucus, the heart rate and flushing responses. In turn, H2 receptors are involved in the control of vascular permeability, gastric acid secretion and airway mucus production.[176,177] H3 receptors are located in the brain and they have been implicated in locomotor activity. Finally, H4 receptors have been associated with histamine induced-chemotaxis, calcium flux in BM mast cells and the migration of tracheal mast cells from the connective tissue to the epithelium [reviewed in [176]].

Similarly to other cells, activation of mast cells also induces *de novo* production of mediators such as cytokines, chemokines and active lipids like eicosanoids. Many studies have shown that the *de novo* production of these mediators can greatly vary depending on the stimuli and the experimental conditions (e.g. IgE-mediated activation of human mast cells is associated with higher production of

eicosanoids than the application of neurogenic peptides or pharmacological stimuli).[143,178]

Human mast cells can produce and release several lipid mediators, including leukotrienes and prostaglandins. Both leukotrienes and prostaglandins are metabolic products of arachidonic acid, derived from membrane phospholipids through the action of phospholipase enzymes.[176] Their lipid structure and the requirement for a single catalytic reaction to become bioactive metabolites allows leukotrienes (e.g. LTC_4 and LTB_4) and prostaglandins (e.g. prostaglandin D_2; PGD_2) to be quickly generated and released by mast cells.[179] Generation of eicosanoids can be induced by activation of c-Kit and/or FcεRI, which elicit a two-phase release of lipid mediators: i) immediate exocytosis of mast cell granules which associated with the synthesis of LTC_4 and PGD_2 and is completed within 10 minutes[180,181] and, ii) a delayed release of PGD_2 alone, occurring 2 to 10 hours after activation.[182] Leukotrienes predominantly exert their effects on the vascular endothelium, promoting the rolling and recruitment of neutrophils through the expression of CD62P (P-selectin) and neutrophil chemotaxis.[143,183,184] In turn, prostaglandins contribute to vascular permeability, chemotaxis of various cells, mucus production and activation of nerve cells.[179,185] Other lipid mediators produced by mast cells include the platelet activation factor (PAF) which is 1000 times more potent than histamine in increasing vascular permeability.[176]

Additionally, mast cells produce a wide spectrum of cytokines, including pro-inflammatory cytokines (e.g. TNF-α, IL-6 and IL-1β), Th2 (IL-4, IL-5 and IL-13) and Th1-skewing cytokines (e.g. IFN-γ, IL-12 and IL-18) and anti-inflammatory cytokines such as tumor growth factor (TGF)-β and IL-10, as well as several chemokines (e.g. CCL5 and CXCL8) involved in the recruitment of immune cells to the site of infection [reviewed in [4,147,176]]. TNF-α represents the major cytokine released by mast cells, both as a preformed and stored mediator and as a newly-synthesized molecule with

multiple functions (e.g. upregulation of endothelial and epithelial adhesion molecules and increase bronchial responsiveness).[146]

Finally, mast cells can also produce antimicrobial peptides known as cathelicidins, which can act as natural antibiotics by directly killing a wide range of microorganisms.[186]

1.2.3. Regulation of mast cell activation

Mast cell activation by different triggers is regulated by several inhibitory mechanisms, which include ligation of receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) (e.g. FcγRIIb, CD300a, Siglec-8, LAIR and SIRP-α), the IL-10 and TGF-β anti-inflammatory cytokines and intracellular signaling molecules that modulate FcεRI-mediated mast-cell activation (e.g. the Lyn negative regulator) [reviewed in [161,187,188]]. Similarly, engagement of the CD200 myeloid cell inhibitory receptor has been shown to inhibit FcεRI-induced mast cell activation,[189] and some mast cells' innate inhibitors like chondroitin sulphate and heparin proteoglycans, have also been shown to hamper secretion of mast cell mediators.[190] Additionally, nitric oxide has also been reported to block FcεRI-induced cytokine secretion through inhibition of Jun,[191] and both the alpha 1-trypsin natural chymase inhibitor and the SLPI secretory protease inhibitor are known to inhibit histamine release by human mast cells.[192] In turn, recent studies have shown the involvement of mitochondria in the regulation of mast cell degranulation due to the requirement of mitochondria translocation to the cell surface for mast cell degranulation. In this regard, the UCP2 (mitochondrial uncoupling protein) protein inhibits mast cell activation and downregulation of Drp1 (dynamin related protein 1), a cytoplasmic protein responsible for mitochondrial fission and translocation, blocks mast cell degranulation.[193-195] Other molecules involved in the regulation of mast cell activation include retinol, β2-

adrenoreceptor agonists, mast cell function-associated antigen (MAFA) and CD84 (SLAMF-5), among other molecules.[187,196-198]

1.2.4. Monitoring of mast cell activation

Following appropriate stimulation, mast cells release most of their granular contents into the surrounding environment. Although these cells can be activated without concomitant degranulation, most physiological stimuli induce the release of mediators stored in the cytoplasmic granules.[199] Exocytosed products become detectable within a few minutes after cell activation and they can be highly specific for mast cells. By contrast, release of *de novo* synthesized mediators upon cell activation requires hours and it is usually less specific, as most cytokines and chemokines produced by mast cells can also be released by other cells.[199] Therefore, monitoring exocytosis of the mast cell granules represents a rapid and convenient mean for evaluating mast cell activation.

At the histological level, occurrence of mast cell degranulation translates into a lack of uniform staining and/or reduced staining of mast cell cytoplasmic granules (decreased granular density or loss of granular contents, reflected by the presence of empty rings around the remains of granular matrices[200]) together with the presence of extracellular granules and diffusely spread granule contents, as detected by means of e.g. immunohistochemical tryptase staining.[201,202] Despite this, for decades the most widely used method to evaluate mast cell activation/degranulation involves the detection of soluble mediators, like histamine, which are released during degranulation.

Several techniques can be used for the *in vitro* measurement of histamine, including competitive enzyme immunoassays and fluorometry, which allow for the detection of biologically active histamine.[199] However, once released into the blood circulation, histamine is rapidly metabolized by histamine methyl transferase leading

to a short half-life (<30 minutes) of histamine that makes the reliable measurement of its serum levels very difficult.[176] Alternatively, histamine and its metabolites can also be measured in urine; however, diurnal variations limit its utility and practical usage as a marker for mast cell activation. [146,176]

In parallel to the release of histamine, secretion of highly-specific mast cell proteases (e.g. tryptase), which comprise more than 90% of the contents of cytoplasmic mast cell granules, also occurs. Thus, monitoring of tryptase serum levels by enzyme-linked immunobsorbent assay (ELISA) techniques, has emerged as a useful marker for mast cell degranulation.[203] Nonetheless, despite tryptase is stored in the granules as a mature active enzyme, protryptase is constitutively secreted by resting mast cells.[146,204] Since most commercially available clinical assays for tryptase recognize both protryptase and mature tryptase, baseline serum tryptase levels consist primarily of secreted protryptase and therefore they more closely reflect the total mast cell burden, rather than mast cell activation.[146,203] New immunoassays, using antibodies which specifically recognize mature tryptase, released simultaneously with histamine, but more slowly disseminated through tissues into the blood than the former, allow for the monitoring of mast cell activation for a relatively long period of time (15 to 120 minutes) after exposure to the stimuli responsible for mast cell degranulation.[203] Assays aiming at the detection of i) the β-hexosaminidase lysosomal enzyme, released simultaneously with histamine and tryptase, but that is not specific for mast cells[199] and, ii) serotonin, a mast cell mediator which has been described to be synthesized upon mast cell activation, have also been used in the literature to assess mast cell degranulation.[205,206]

Except for the histological and morphological techniques, none of the former approaches allows for direct assessment of mast cell activation at the single cell level; furthermore, all such assays monitor relatively late events occurring during mast cell activation/degranulation. Other techniques based on the detection of Annexin-V binding to phosphatidylserine residues at the external leaflet of the plasma membrane

of mast cells, allow assessment of mast cell activation in individual cells.[199] Additionally, activation of mast cells by IgE-dependent stimuli or other agonists, is also associated with significant changes in the immunophenotypic profile of mast cells, such changes involving the expression of β2 integrins, cytokine receptors, complement receptors, and members of the tetraspanin antigen family;[104] some of these changes reflect mast cell degranulation, such as surface membrane expression of the lysosomal-associated membrane proteins LAMP-1 (CD107a) and LAMP-2 (CD107b), together with increased reactivity for LAMP-3 (CD63) on the plasma membrane.[104,207,208] Similarly to CD63, surface membrane expression of CD203c (ectonucleotide pyrophosphatase/ phosphodiesterase 3), is also significantly up-regulated upon IgE activation, as a result of degranulation.[104,120] However, the kinetics of CD63 upregulation and its sensitivity to inhibitors and activators of FcεRI-mediated signaling, differ significantly from those of CD203c, suggesting that these two molecules are linked to two distinct cell activation mechanisms.[209] More recently, surface expression of CD200R1 has also been reported to be an activation/degranulation marker, as it was upregulated in the plasma membrane of mouse mast cells upon stimulation with anti-IgE.[210]

Immunophenotypic changes involving other cell surface antigens reflect activation of mast cells by IgE and/or other agonists, more than the degranulation process itself. Among other, these include upregulation of the CD69 early-activation cell surface antigen,[104,139,211] expression of FcγRI (CD64) induced upon exposure to IFNγ,[148] and reactivity for MHC class II associated molecules (e.g. HLA-DR), which are not detected in resting MC, but present on mast cells isolated from pathogen-infected tissues and/or mast cells stimulated by TNF, IFNγ or LPS.[212-214]

1.3. The physiological role of mast cells

Mast cells are strategically located at many sites of antigen entry (including the skin, lung and gastrointestinal tract) where they can detect and initiate responses

against invading microbes, being able to shape the immune response and act as a bridge between innate and acquired immunity.[3,4]

1.3.1. The functional role of mast cells in the innate immune response

Mast cells have been implicated in host defense against a large number of pathogens, including bacteria, parasites, viruses and fungus.[3] Accordingly, these cells are well known to play an important role in innate immune responses to bacterial infections, as they are susceptible of being activated by bacterial constituents via TLRs (e.g. TLR2 and TLR4), CD48 and G-protein coupled receptors for peptides that are upregulated during bacterial infection (e.g. endothelin-1 or neurotensin-1).[166,215,216] Upon activation, the release of TNF-α, LTC$_4$ and LTB$_4$ contributes to the recruitment of neutrophils and clearance of infection.[217-219] Furthermore, mast cells also show phagocytic properties[220] and exhibit direct bactericidal activity (e.g. through release of cathelicidins[186] and proteases[221]), at the same time they are able to kill bacteria by entrapping them in extracellular structures composed of DNA, histones, tryptase and cathelicidin antimicrobial peptides, the mast cell extracellular traps (MCETs).[222]

Additionally, these cells have been long known to play a role in host defense responses against parasitic infections.[223] Their preferential location in organs targeted by parasites (e.g. skin and gut), together with the expression of key receptors (e.g. TLR1, TLR4 and TLR9) which allow mast cells to directly react to parasitic stimuli, contribute to control parasite infections (e.g. intracellular protozoan or intestinal helminth parasites) through a wide range of mechanisms, including also recruitment of effector immune cells like neutrophils, regulation of gut permeability and expulsion of parasites.[215]

Unlike bacterial and parasitic infections, contribution of mast cells to antiviral immunity remains largely unknown. However, at present it is well established that mast cells can be infected by several virus (e.g. HIV - human immunodeficiency

virus -, dengue virus, cytomegalovirus and adenovirus) and they can also be activated through TLR-3 and TLR-7 receptors, following interaction with double-stranded and single-stranded viral RNA, respectively.[4,224] In addition, some viral products can function as superantigens and activate mast cells by interacting with the V_H3 region of IgE bound to FcεRI.[4] Viral activation induces a unique profile of mediator release, dominated by the production of cytokines (e.g. IL-1β, IL-6), chemokines (e.g. CCL3, CCL4, CCL5 and CXCL8) and type I interferons (IFN-α and IFN-β), that play a key role in antiviral responses, allowing for the recruitment of effector cytoxic cells such as CD8$^+$ T cells and NK cells.[4,224-226]

The role of mast cells in antifungal defense is an almost unexplored area of research, even though mast cells express several receptors and mediators known to be involved in antifungal responses [reviewed in [215]]. Mast cells have been described to respond to yeast cell wall zymosan and peptidoglycan by releasing cysteinyl leukotrienes and producing reactive oxygen species (ROS).[215] These responses are mediated by several receptors, including TLR2[157] and dectin-1, the β-glucan receptor for the C-type lectin family which mediates the response to zymosan by human mast cells.[227]

1.3.2. Functional role of mast cells in the adaptive immune response

Upon stimulation, mast cells provide important signals that help the generation of an appropriate adaptive immune response to infection, at the same time they support the early recovery from infection and allow for a proper memory response in case a particular pathogen is encountered again.[143] These signals have the potential to influence several steps of adaptive immune responses, from antigen presentation to T cell activation and homing to target tissues. Accordingly, activated mast cells express both MHC class I and II proteins and they can process and present antigens to T cells.[228-230] Furthermore, they can indirectly present antigens to T cells

through internalization of antigen-bound FcɛRI through a mechanism which is independent of MHC II expression, involving apoptosis and phagocytosis of mast cells by other antigen presenting cells (APC).[231] In addition, mast cells also express several costimulatory/inhibitory receptors and adhesion molecules; among others these include members of the B$_7$ family of costimulatory receptors (CD80, CD86), ICAM-1 (CD54), ICAM-3 (CD50) and β-integrins like LFA-1 (CD11a/CD18), which enable mast cells to interact with endothelial cells, lymphocytes and fibroblasts, further supporting the role of these cells as APCs [reviewed in [4,212]].

In addition to presenting antigens themselves, mast cell products can directly modulate dendritic cell activation and antigen presentation. As an example histamine has been shown to promote antigen uptake and cross-presentation[232] together with up-regulation of co-stimulatory molecules required for T-cell activation on APC.[233] Furthermore, mast cell mediators can promote dendritic cells to acquire a Th2 cell-inducing phenotype[234] and they are capable of directly influencing T cell recruitment to the sites of infection through the release of multiple chemotactic factors (e.g. IL-6, CCL2, CCL3, CCL4, CCL5 and CCL20) or by up-regulating the expression of several adhesion molecules (e.g. E-selectin, ICAM-1 or VCAM-1) on endothelial cells [reviewed in [235]]. Furthermore, mast cells are a source of polarizing cytokines such as IL-2, IL-4, IL-12, IL-23, IFN-γ, TGF-β and IL-6 [reviewed in [236]] and therefore they can potentially induce differentiation of T cells.[4] Of note, mast cells also express CD154 (CD40L), a molecule that induces immunoglobulin class switch upon interaction with B cells, and release several cytokines (i.e. IL-4, IL-5, IL-6 and IL-13) which act as B cell growth and differentiation factors.[235]

Mast cells not only promote the initiation and development of adaptive immune responses, but they also contribute to limit the duration and magnitude of such responses, as it has been demonstrated that they are important for mediating CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T cell-dependent tolerance in a murine skin transplant model.[237,238]

1.1.3.3. Other physiological roles of mast cells

Apart from being prominently involved in the immune system, mast cells orchestrate several other biological systems and maintain the homeostasis in the body.[4] Accordingly, mast cells are known to influence several stages of wound healing, including the initial inflammatory response, re-epithelialization and revascularization of the damaged tissue and the deposition of collagen and remodeling of the extracellular matrix.[239] Moreover, they also play relevant roles in the homeostasis of organs that undergo continuous growth and remodeling, such as hair follicles and bones.[4] In turn, growing evidence of bidirectional interaction of mast cells and neurons suggest that both cells represent a functional unit. Accordingly, mast cell products such as histamine, serotonin and tryptase, can influence the activity of sensory neurons; conversely, mast cells can be activated by products released by neurons such as the calcitonin gene related peptide (CGRP) or substance P, allowing these cells to cooperate in order to influence various physiological conditions, including the stress response.[4]

2. ROLE OF NORMAL/REACTIVE MAST CELLS IN THE PATHOPHYSIOLOGY OF ALLERGIC AND NON-ALLERGIC DISEASES

Apart from their key role in multiple physiological conditions, normal/reactive mast cells are involved in the pathogenesis of various chronic allergic/inflammatory disorders and cancers. The most widely studied example of the functional deregulation of mast cells and their physiological role relates to the pathophysiology of allergic diseases, including IgE-mediated hypersensitivity reactions in airways, skin, and the gastrointestinal (GI) tract such as asthma, allergic rhinitis, atopic dermatitis and food allergy.[3] These responses result from the activation of FcεRI by a polyvalent allergen

recognized by bound IgE, leading to mast cell activation and the release of both stored and newly-synthesized mediators into the local environment.[240] Early mast cell released mediators include histamine, tryptase, LTC_4, PGD_2, PAF, CCL2, IL-13, vascular endothelial growth factor A (VEGF-A) and TNF-α. Such mediators cause most of the pathological symptoms and signs associated with allergy, as they have immediate effects on epithelial, smooth muscle and endothelial cells and nerves, leading to increased epithelial permeability, mucus production, smooth muscle contraction, vasodilation and neurogenic signals.[3,161] A classical example of an acute allergic response mediated by the release of early mast cell mediators is anaphylaxis.[4] However, allergic diseases do not exclusively consist of this "early phase reaction", but they also include subsequent events involved in a "late phase reaction", which typically develops 2-6h after exposure to the allergen. This "late phase reaction" is directed both by a prolonged release of mast cell mediators and the activation of newly-arrived leukocytes and tissue-resident cells, in response to mast cell mediators (e.g. TNF-α, LTB_4, IL-5, IL-8 and CCL2) which induce i) the recruitment and local activation of neutrophils, eosinophils and basophils, among other cells, and ii) the initiation of an adaptive immune response.[161,241] Persistent inflammation induced by prolonged or repetitive exposure to specific allergens, leads to a "chronic allergic inflammation", typically characterized by the presence of large numbers of innate and acquired immune cells at the affected site and by changes in the extracellular matrix, leading to tissue remodeling and fibrosis [reviewed in [241]].

Deregulated activation of mast cells through IgE-independent mechanisms, linked to IgG or IgM autoantibodies, immune complexes and TLRs, have been implicated in several autoimmune disorders, including psoriasis, rheumatoid arthritis and multiple sclerosis.[242] Although the demonstration of the involvement of mast cells in many of these disorders is mainly based on the observation of mast cell hyperplasia and/or increased mast cell products at sites of tissue injury, direct participation of mast cells in the pathogenesis of some of these diseases has also been reported.[4,187,242]

As an example, mast cells from multiple sclerosis patients have been reported to release cytokines/chemokines which selectively i) induce T cell and macrophage recruitment and activation, ii) present myelin antigens to T cells, iii) disrupt the blood-brain barrier and, iv) facilitate the entry of T cells that are sensitized to myelin basic protein and damage myelin; in turn, activated T-cells lead to the release of myelin fragments that may further stimulate secretion of tryptase, which may in turn enhance demyelination and induce further inflammation through stimulation of mast cell protease-activated receptors (PAR) [reviewed in [187,243]].

Increasing evidences suggest that mast cells play a relevant role in the pathophysiology of several cardiovascular diseases, such as atherosclerosis, the major underlying cause of acute cardiovascular syndromes (e.g. myocardial infarction and stroke) characterized by a lipid-driven chronic inflammatory process.[4,244] Accordingly, greater numbers of mast cells are found in atherosclerotic plaques.[245] These mast cells produce and/or release mediators like histamine, tryptase, chymase and cytokines such as TNF-α, IFN-γ, MCP-1, IL-6 and IL-8 which contribute to the development, progression and destabilization of atherosclerotic lesions.[244] Additionally, mast cell activation in atherosclerosis has been demonstrated to promote lipid uptake by macrophages, lipoprotein retention within the lesion, apoptosis of various vascular cells, leukocyte recruitment, vascular leakage and intraplaque hemorrhage, all resulting in the progression and destabilization of the atherosclerotic plaques [reviewed in [244]]. Other examples of the impact of mast cells in cardiovascular disease include the Kounis syndrome, characterized by the concurrence of an acute coronary syndrome and a mast cell activation syndrome, associated with allergic and hypersensitivity reactions which include anaphylactic or anaphylactoid insults, as a result of the effect of inflammatory mediators released during mast cell activation; such insults may ultimately lead to *angina pectoris* and myocardial infarction.[246,247]

Mast cells have long been recognized to infiltrate the interface between tumors and healthy tissues.[248,249] However, the impact of mast cell accumulation in the tumor

periphery still remains controversial as some studies reported that mast cells might promote tumor growth (e.g. in rectal cancer tumors), whereas others support an inhibitory growth effect for these cells (e.g. in breast cancer).[248,249] Despite such apparent discrepancies, several functions have been suggested for mast cells in tumor growth through e.g. an increased angiogenesis.[250] In fact, mast cells express a wide range of pro-angiogenic factors and they are a major source of proteases which can act on the surrounding extracellular matrix and therefore play an important role in tissue remodeling during tumor growth.[248] Moreover, mast cells have also been reported to affect tumor growth by mast-cell mediated cytotoxicity and immune regulation through both recruitment of immune cells and/or immunosuppression.[248,249]

3. CLONAL MAST CELLS AND MASTOCYTOSIS

Homeostatic regulation and control of tissue mast cell numbers is a complex process which involves an intricate network of factors that affect cell proliferation, differentiation and survival. Deregulation of these processes leads to an altered (e.g. increased) tissue mast cell load which frequently results from changes in the tissue microenvironment (e.g. B-cell chronic lymphoproliferative disorders, chronic atopic disease, or helminthic/bacterial infection), leading to a reactive mast cell hyperplasia.[5,109] However, a pathological accumulation of clonal mast cells may also occur, with both systemic and local consequences, as a result of an increased mast cell burden and the potentially augmented release of mast cell-associated mediators.[5] These later cases represent a rare group of disorders, with an estimated prevalence of 1-5 individuals per 10,000 individuals,[251] which are categorized under the general term of "mastocytosis". Consequently, mastocytosis includes a very heterogeneous group of diseases, that may or may not have a familial component, which can occur at any age, and show a clinical course which ranges from "mild" disorders with a normal life

expectancy to "highly aggressive" malignant disease.[7,252] Furthermore, the clinical features of clonal mast cell disorders include a wide range of symptoms, which are frequently related to the release of mast cell mediators (e.g. pruritus, flushing, nausea, vomiting, diarrhea, abdominal pain and vascular instability, among other symptoms) and/or the involvement of distinct organs/tissues (e.g. skin, bone marrow, gastrointestinal (GI) tract, liver, spleen or the lymph nodes).[5]

3.1. The pathogenesis of mastocytosis

Based on the recognition that SCF is the main mast cell growth factor, Nagata *et al.*[253] identified an activating point mutation in the peripheral blood of 4 patients with a systemic form of mastocytosis who showed predominant myelodysplastic features. Such point mutation, consisted of a substitution of a valine by an aspartic acid in the codon 816 of the catalytic domain of c-Kit (D816V *KIT* mutation). This mutation disrupts the hydrogen bond between residues D816 and N819, which destabilizes the inactive conformation of the kinase domain of c-Kit,[254] leading to constitutive c-Kit autophosphorylation and deregulation of mast cell function (e.g. increased proliferation).[255,256] Subsequent studies reported that more than 90% of adults with systemic mastocytosis carry this mutation.[257] Additionally, other less frequent (<5%) somatic *KIT* mutations have also been identified in patients with mastocytosis (Table 7). Such mutations cluster in relatively small regions of the molecule, most frequently at exons 11 and 17, leading to aminoacid changes at the juxtamembrane domain and the tyrosine kinase (TK) 2 domain of c-Kit, respectively (e.g. V560G, R815K, D816Y, D816H, D816F, among other mutations).[26] Less frequently, other *KIT* mutations are detected at exons 8 and 9, which code for the extracellular domain of c-Kit (e.g. K509I) (Table 7).[26]

Despite controversial results exist in the literature as regards mastocytosis with a childhood-onset, recent studies suggest that they also represent a mast cell disease associated with either germline or acquired activating *KIT* mutations.[258,259]

However, the *KIT* genotype varies significantly with the age of onset,[260] since the D816V *KIT* mutation seems to be less frequent in childhood *vs.* adult onset mastocytosis (35% *vs.* >90%, respectively), whereas mutations outside exon 17, specially activating mutations at exons 8 and 9, are more frequently found among pediatric (cutaneous) forms of the disease (Table 7).[257,258]

Table 7. *KIT* mutations reported in patients with mastocytosis. [Modified and updated from Orfao *et al.*[26]]

c-Kit DOMAIN	EXON	MUTATION	EFFECT	FREQUENCY (%)	COMMENTS	REFERENCES
Extra-cellular	8	D419del	Unknown	<5	Familial SM	Hartman (2005)[261]
		FF419ins	Unknown	<5	Pediatric CM	Bodemer (2010)[258]
		T417_D419del insY	Activating	<5	Pediatric CM	Bodemer (2010)[258]
		C443Y	Activating	<5	Pediatric CM	Bodemer (2010)[258]
		S476I	Activating	<5	Pediatric CM	Bodemer (2010)[258]
	9	S501_A502dup	Unknown	<5	SM	Georgin-Lavialle (2012)[262]
		A502_Y503dup	Unknown	<5	SM	Mital (2011)[263]
		N505_F508dup	Unknown	<5	Pediatric CM	Bodemer (2010)[258]
		K509I	Unknown	<5	Familial SM	Zhang (2006)[264]
Trans-membrane	10	F522C	Activating	<5	SM	Akin (2004)[265]
		A533D	Activating	<5	Familial SM	Tang (2004)[266]
Juxta-membrane	11	V559I	Activating	<5	SM	Nakagomi (2007)[267]
		V560G	Activating	<5	SM	Furitsu (1993)[255] Buttner (1998)[268]
		D572A	Activating	<5	Pediatric CM	Bodemer (2010)[258]
TK1	13	V654A	Unknown	<5	SM	Spector (2012)[269]
TK2 Activation loop	17	R815K	Unknown	<5	Pediatric CM	Sotlar (2003)[270]
		D816A	Unknown	<5	SM-AML	Yabe (2012)[271]
		D816V	Activating	>90	Adult SM	Garcia-Montero (2006)[257]
		D816Y	Activating	<5	SM	Longley (1999)[272]
		D816H	Unknown	<5	SM-AML	Pullarkat (2000)[273]
		D816F	Activating	<5	SM	Longley (1999)[272]
		D816I	Activating	<5	Pediatric CM	Bodemer (2010)[258]
		I817V	Unknown	<5	SM	Garcia-Montero (2006)[257]
		V815_I816ins	Unknown	<5	SM	Garcia-Montero (2006)[257]
		D820G	Unknown	<5	SM	Pignon (1997)[274]
		N822I	Activating	<5	Familial SM	Wasag (2011)[275]
		E839K	Inactivating	<5	CM	Longley (1999)[272]

SM, systemic mastocytosis; CM, cutaneous mastocytosis; TK1, c-Kit tyrosine kinase domain 1; TK2, c-Kit tyrosine kinase domain 2; AML, acute myeloid leukemia.

Other oncogenic mutations, which cosegregate with the *KIT* D816V mutation, have been recently identified in small subsets of patients.[276] Despite most of these mutations are not specific for mastocytosis and their impact in the pathogenesis and prognosis of the disease remains largely unknown, some associations with the clinical features of the disease have been suggested.[276] Accordingly, mutations in the *TET2* (*TET* oncogene family member 2) tumor suppressor gene, previously described in myeloid neoplasms,[277] have been reported to be present in 29% (n=12/42 patients) of mastocytosis patients with systemic forms of the disease, specially among those patients who also display monocytosis.[278] Furthermore, the V617F *JAK2* mutation typically detected in Philadelphia-chromosome-negative myeloproliferative neoplasms (MPN) (e.g. primary myelofibrosis, polycythemia vera and essential thrombocythemia),[279] has also been identified in highly purified mast cells from 4 patients with systemic mastocytosis (SM) carrying an associated chronic idiopathic myelofibrosis.[280] More recently *NRAS* activating mutations have also been reported in 2 out of 8 patients with advanced forms of SM, while such mutations were systematically absent in 36 patients with indolent forms of the disease.[281] Interestingly, the Ras/MAPK pathway is a c-Kit downstream signaling pathway, which is important in the regulation of mast cell proliferation and survival (Figure 1C),[26] suggesting that simultaneous presence of the two activating mutations could have a synergistic impact on the expansion of this cell compartment, and therefore, also in the severity of the disease.[281] A similar association has been recently reported between the V654A *KIT* mutation previously described in gastrointestinal stromal tumors (GIST), and the L188F missense mutation involving the *MS4A2* gene that codes for the β-chain of the high-affinity tetrameric cell-surface IgE (FcεRI) receptor in a patient with an aggressive form of SM who tested negative for the more frequent D816V *KIT* mutation.[269]

Finally, two recent reports suggest that mast cell disorders associated with *KIT* mutations may be influenced by the genetic background of the individual, since simultaneous presence of the Q576R gain-of-function polymorphism in the

cytoplasmic domain of the IL-4α receptor subunit (IL-4Rα), appears to be associated with less extensive mast cell involvement and disease restricted to the skin.[282] Conversely, the -1112C/T *IL-13* promoter gene polymorphism, known to be associated with increased *IL-13* transcription, was found to be significantly more frequent among patients with systemic variants of the disease, adult-onset mastocytosis and higher serum tryptase levels.[283]

All the above observations support the hypothesis that mastocytosis most likely results from the presence of a constitutively activated SCF/c-Kit pathway in clonal mast cells from adult patients and some children, associated with other secondary (or coexisting) genetic events, giving rise to distinct clinical variants of the disease.[5]

3.1.1. Impact of the KIT mutation in mastocytosis

Despite several mutations and polymorphisms have been described in mastocytosis, the presence of a *KIT* activating mutation seems to be the common denominator to the great majority of patients, specially for those with an adult onset and systemic variants of the disease.[26,257] Activation of the c-Kit receptor and its downstream pathways in normal mast cells, leads to increased cell proliferation, survival, degranulation and mediator release, as well as to changes in mast cell adhesion and migration properties;[26,30,35] consequently, the presence of a *KIT* mutation leading to constitutive ligand-independent activation of the receptor, could be expected to severely affect these mast cell functions.[26]

3.1.1.1. Proliferation and survival of KIT mutated mast cells

Under physiological conditions, activation of c-Kit following SCF binding induces mast cell proliferation and survival. Since the majority of patients with mastocytosis carry an activating mutation - most frequently the D816V mutation[257] -, it

could be expected that these functions would be affected in their clonal mast cells.[26] In fact, gain-of-function mutations of *KIT* have been classically described to induce mast cell proliferation,[284-286] as a consequence of the activation of several downstream pathways which are involved in controlling cell proliferation and survival (e.g. PI3K, MAPK and PKC) (Figure 1C).[52] In line with this, studies performed in the SCF-independent human mast cell-1 (HMC-1) cell line, which carries two activating *KIT* mutations (i.e. D816V and V560G), found that constitutive activation of c-Kit and its downstream pathways leads to increased proliferation in a NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells)-dependent way.[287] Therefore, constitutive activation of c-Kit results in increased expression of cyclin D3, through the activation of NFκB and its translocation to the nucleus; such increased cyclin D3 expression leads to phosphorylation of Rb (pRb; phosphorylated retinoblastoma protein) and S-phase promotion in the activated clonal mast cells.[26,287]

Moreover, activation of SCF/c-Kit-associated signaling pathways is also known to increase mast cell survival. As an example, SCF-mediated activation of the PI3K/Akt pathway leads to the inhibition of the Bad pro-apoptotic protein (Figure 1C).[26] Regulation of the anti-apoptotic activity of c-Kit exerted through the PI3K/Akt pathway is controlled by phosphorylation of residue Y721 of c-Kit at its kinase insert domain;[26,50] thus, conformational changes induced by mutations at the activation loop (e.g. D816V) of *KIT* that cause activation of the PI3K pathway, may also contribute to mast cell transformation through an enhanced survival.[26] In this regard, mTOR (mammalian target of rapamycin), a downstream serine/threonine kinase target of Akt in the PI3K pathway, is also constitutively activated in HMC-1 cells carrying the D816V mutation.[288] Also, D816V$^+$ mast cells isolated from patients diagnosed with systemic variants of mastocytosis, but not normal mast cells, are sensitive to rapamycin, which leads to the inhibition of survival of clonal mast cells.[288,289] Furthermore, neoplastic mast cells from several cell lines and mastocytosis patients, carrying different

activating *KIT* mutations, have been shown to display constitutive activation of STAT5, which contributes to both the growth and survival of clonal mast cells.[290-293]

Overall, due to these effects of activating c-Kit mutations on mast cell proliferation and/or survival, they typically lead to an increase in mast cell numbers, associated with an abnormal accumulation of these cells in one or more affected tissues.[294]

3.1.1.2. High-throughput gene expression profiling

High-throughput gene expression profiling has emerged over the last decades as a powerful tool for the advancement of biomedical research.[295] Rapid progress in the development of cDNA microarray technology which allow for simultaneous determination of mRNA expression levels of thousands of genes, has largely contributed to better understand the molecular mechanisms underlying normal and altered biological processes in multiple neoplastic and non-neoplastic disease conditions.[296-300] Furthermore, it has led to the identification of new gene expression programs and pathways, with a significant impact in patient diagnosis, classification, prognosis and the evaluation/prediction of the response to therapy.[300,301] More recently, high-throughput gene expression profiling studies performed on highly-purified cell populations allowed for the study of very infrequent cell subsets; this has increased sensitivity of the technique, overcoming important methodological limitations associated with both the study of specific cell population within an heterogeneous cellular background and the variability associated with different levels of infiltration by tumor cells in a sample.[302-304]

Despite the potential value of high-throughput gene expression microarray analyses for better understanding the molecular basis of clinically heterogeneous diseases few reports exist in the literature in which gene expression profiling has been assessed in mastocytosis. Furthermore, in none of them highly-purified mast cells were analyzed.[305-307] In an early study en 2003, D'Ambrosio *et al.*[305]

identified 168 significantly deregulated genes (either up- or down-regulated) in BM mononuclear cells from patients with systemic mastocytosis (n=8) *vs.* healthy controls (n=5). Up-regulated genes were associated with proteins contained in mast cell granules (e.g. α- and β-Tryptase and CPA), transcription factors, cell-cycle related genes and genes involved in neoplastic transformation and apoptosis.[305] More recently, in two consecutive studies Niedoszytko *et al.*,[306,307] analyzed the gene expression profile (GEP) of non-sorted whole peripheral blood samples from patients with indolent forms of systemic mastocytosis with (n=12) and without (n=10) previous history of insect venom anaphylaxis, and compared them with those of 43 healthy controls. Overall, these authors found abnormal GEPs, which involved the MAPK, JAK/STAT and p53 cancer-associated signaling pathways, together with cell cycle and apoptosis-related genes. Furthermore, a GEP consisting of a combination of 29 genes was identified which could contribute for the diagnosis of these patients.[307] In addition, distinct GEP (n=104 genes) were reported for patients with and without prior history of anaphylaxis; in this later set of genes, genes involved in pathways favoring cell differentiation over cell proliferation in patients with insect venom anaphylaxis, were included, suggesting that mast cell differentiation could be a critical determinant of the sensitivity to anaphylaxis, at the same time, gene expression profiling would be a useful tool for the identification of patients who are at risk of insect venom anaphylaxis.[306]
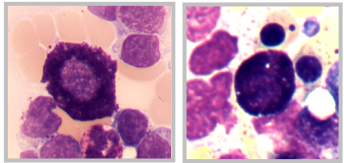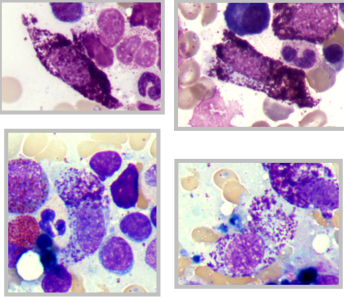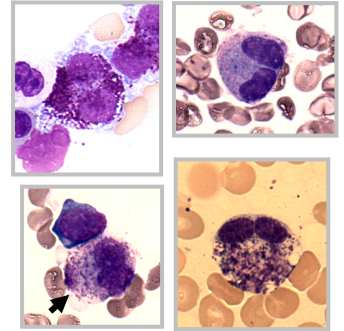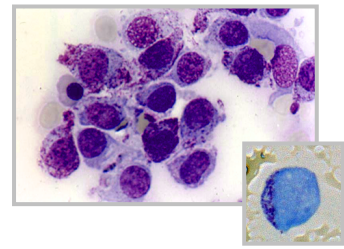
3.1.1.3. The morphology of KIT mutated mast cells

Physiological activation of mast cells is also accompanied by the engagement of exocytosis, cell migration and/or adhesion to various substrates. These processes are frequently associated with morphological changes in the cells involved, and they are highly dependent on the activity of cytoskeleton proteins.[308] Accordingly, early studies by Vosseller *el al.* in 1997 using scanning electron microscopy, reported changes in the membrane morphology of mouse BM mast cells upon activation of c-Kit

with SCF, for 30 min. These authors described unstimulated mast cells as round and compact cells, whereas a dramatic structural reorganization occurred upon activation: membrane folds extended radially and the cells appeared to spread.[53] Furthermore, these c-Kit dependent morphologic changes required actin reorganization, triggered by activation of both PI3K and Rac.[53,308] More recently, SWAP-70 (switch-associated protein 70), a PI3K-dependent guanidine nucleotide exchange factor (GEF) that binds phosphatidylinositol-3,4,5-trisphosphate (PIP3), the second messenger product generated by PI3K, was also described to be implicated in the interaction with F-actin (filamentous actin).[309] Accordingly, SWAP-70 contributes to regulate polymerization and/or depolymerization of actin, a process that is required for the rearrangement of the actin cytoskeleton, and consequently also for changing cell shapes.[310] During mast cell activation, SWAP-70 also targets activated Rac to the cell periphery, a process which is also necessary for actin rearrangements.[310] Fyn (a SFK which is activated upon SCF-induced mast cell activation) has also been recently described to be required for c-Kit mediated activation of PI3K/Rac2-dependent pathways involved in the reorganization and polarization F-actin during spreading and lamellipodia formation, therefore leading to morphologic changes of mast cells.[311]

Interestingly, activation of PI3K, similarly to other SCF/c-Kit downstream pathways (p38α[44,47] MAPK and the JAK/STAT[68,69]), also plays an important role in the regulation of mast cell differentiation.[50-54] Since activation of c-Kit and its downstream pathways are both involved in cytoskeleton rearrangements and regulation of mast cell differentiation, the presence of a *KIT* mutation leading to constitutive activation of this receptor, would potentially have also an important impact on the morphologic appearance of clonal mast cells. In line with this hypothesis, *KIT*-mutated mast cells exhibit altered but distinct cytomorphological properties, which correlate with the different stages of mast cell maturation.[312] Such altered cytomorphological features of *KIT*-mutated mast cells have been classified into four separate morphological sub-groups by Sperr *et al.* in 2001 (Table 8).[312]

Table 8. Distinct subtypes of clonal mast cells as defined by morphologic examination of BM smears stained with May-Grünwald-Giemsa and toluidine blue (isolated metachromatic blast). [Modified from Sperr et al. [312]]

Morphological subtype	Main morphological features	Illustrating examples
TYPICAL TISSUE MAST CELL (MATURE)	• Round or oval cell; • Small or medium size; • Round nucleus with central position; • Condensed chromatin; • Low N/C ratio; • Well granulated cytoplasm;	

ATYPICAL MAST CELL TYPE I	Two or three of the following:
	  • Prominent surface projections;
	  • Oval nuclei with excentric position;
	  • Hypogranulated cytoplasm with focal granule accumulations with or without granule fusions;	

ATYPICAL MAST CELL TYPE II (PROMASTOCYTE)	• Variable shape of cell; • Bi- or polylobed nuclei; • Nucleoli may be present; • Cytoplasm often hypogranulated; • Low N/C ratio (more mature) or high N/C ratio (more immature); • Nuclear chromatin condensed (mature) or fine (immature);	
METACHROMATIC BLAST (IMMATURE)	• Blast-like morphology; • Nuclear pattern as blasts; • Prominent nucleoli; • Few metachromatic granules;	

N/C, nucleus/cytoplasm.

Thus, Wright-Giemsa stained BM mast cells from patients with clonal mast cell disorders, exhibit morphologic features which range from those of a metachromatic granulated blast (usually detected in an early phase of mastopoiesis) and an immature atypical mast cell with bi- or multilobed nuclei (promastocyte or

atypical mast cell type II), to more mature cells which may show an atypical morphology with prominent surface projections, frequently spindle-shaped, with an hypogranulated cytoplasm and/or an oval excentric nucleus (atypical mast cell type I) and typical mature (tissue) mast cell features, consisting of round or oval shaped cells with a round central nucleus and a well granulated cytoplasm (Table 8).[312]

3.1.1.4. Histopathological alterations associated with KIT-mutated mast cells

Constitutive activation of c-Kit on *KIT*-mutated clonal mast cells leads to increased proliferation and survival, as well as morphological changes.[26,312] Altogether, these altered processes are considered to be responsible for an abnormal accumulation of morphologically atypical mast cells in one or more different tissues, ultimately leading to modification of the histological features of the affected tissues.[313] Therefore, biopsies from BM and other affected organs from patients with clonal mast cell disorders, typically show dense, focal and/or diffuse mast cell infiltrates; this is in contrast to cases with reactive mast cell hyperplasia, including myelodysplastic syndromes, which are typically characterized by a diffuse increase in isolated mature, round or spindle-shaped, metachromatic mast cells which are loosely scattered throughout the tissue and do not form dense focal infiltrates, even in states of marked hyperplasia.[313] In BM biopsies, these multifocal, dense aggregates of clonal mast cell are mostly localized in the peritrabecular and/or perivascular areas, typically with a spindle-shaped morphology.[313] Furthermore, in some cases these infiltrates also contain variable numbers of lymphocytes (both B and T cells), plasma cells, eosinophils, histiocytes and/or fibroblasts, together with reticulin fibers, accompanied in specific cases by marked collagen fibrosis.[7,313]

Depending on the number and distribution of mast cells in the BM, different histological patterns can be observed, from increased numbers of interstitial mast cells without evidence of compact mast cell aggregates, to focal infiltrates and

cellular dense mast cell aggregates with a diffuse pattern of infiltration by mast cells that replaces the normal hematopoietic BM tissue (Figure 3).[314]
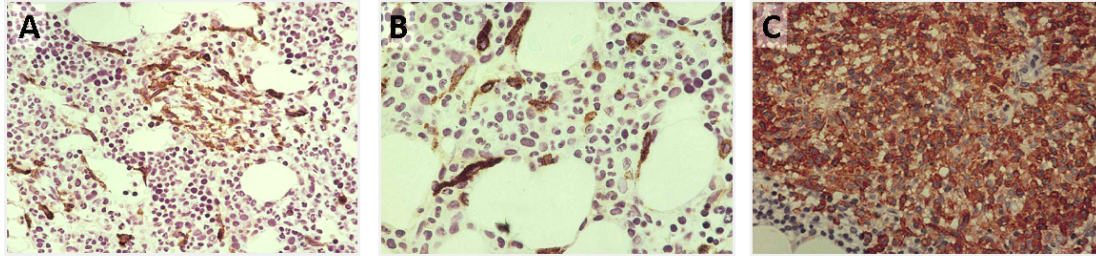

Figure 3. Immunohistochemical tryptase staining of bone marrow (BM) biopsies illustrating the distinct patterns of infiltration observed in mastocytosis patients. In Panel A focal BM infiltration is shown, whereas diffuse interstitial and cellular/dense patterns of infiltration are depicted in Panels B and C, respectively. [Modified from: Horny et al.[313]]

3.1.1.5. Migration and tissue/organ involvement by KIT-mutated mast cells

Currently, it is well-established that mast cell migration to peripheral tissues is also a SCF/c-Kit mediated process.[26,128,315] Accordingly, activation of c-Kit has been shown to be involved in PI3K-dependent mast cell adhesion to the extracellular matrix through fibronectin, due to the activation of fibronectin receptors on these cells.[131] Furthermore, recent data suggests that SCF can also regulate mast cell chemotaxis mediated by the CCL2/CCR2 axis, which plays a central role in antigen-induced recruitment of mast cells to the lung.[316] In line with this, a recent study by Hu et al.[48] identified P38α, a c-Kit downstream MAPK (Figure 1C), as an important factor in the regulation of SCF-induced migration of fully differentiated mast cells, because deletion of P38α leaded to reduced numbers of mast cells in certain tissues. Moreover, constitutive activation of c-Kit disrupts the physiological mechanisms responsible for the regulation of these migration-related processes, and enhances of the chemotaxis of CD117^{+} cells, which therefore contributes to the abnormal pattern of accumulation of mast cells in distinct tissues/organs, due to the disordered cell trafficking.[317] Patients with clonal mast cell disorders show increased numbers of morphologically

atypical mast cell infiltrates in different tissues/organs, including the spleen and liver, where mast cells are virtually absent under physiological conditions.[313] Such massive mast cell infiltration may ultimately lead to organ dysfunction, and potentially also some of the symptoms mastocytosis patients display, e.g. constitutional symptoms like weakness, fatigue, night sweats or weight loss.[294] Interestingly, even among those patients that share the same activating *KIT* mutation (i.e. D816V), variable patterns of involvement of different tissues can be observed (e.g. skin, BM, spleen, liver, lymph node and/or peripheral blood), suggesting that factors other than the *KIT* mutation itself, are involved in clonal mast cell homing to tissues. Nevertheless, the exact mechanisms underlying this heterogeneity remain largely unknown.[26]

3.1.1.6. Systemic manifestations due activation of clonal mast cells

Human mast cell degranulation is mainly driven through binding of IgE/antigen immunocomplexes to the immunoglobulin E high-affinity receptor (FcεRI) on the surface membrane of mast cells. Despite this, activation of c-Kit substantially potentiates antigen-mediated mast cell degranulation and cytokine production.[26,318] Accordingly, in the presence of antigen/IgE immunocomplexes, SCF-mediated c-Kit signaling activates PLCγ (Figure 1C), and ultimately leads to calcium mobilization and mast cell degranulation.[318] This synergistic effect of c-Kit is mediated by NTAL (linker for activation of T cells family, member 2), a protein that acts as a pivotal link between the signaling cascades following c-Kit activation and cross-linking of FcεRI.[319] Therefore, constitutive ligand-independent *KIT*-activating mutations favor an enhanced mast cell response against distinct stimuli (e.g. antigen or physical stimuli) within the mast cell microenvironment, leading to the release of different mediators which may even become systemically detected (e.g. higher serum total baseline tryptase levels or histamine levels).[26] Since mast cell mediators play very different physiological roles (Table 6), constitutive activation of clonal mast cells, driven by mutated c-Kit, leading to increase release of distinct mediators, ultimately results on a wide array of variable

clinical symptoms.[26] Accordingly, patients with systemic clonal mast cell disorders may show one or several mediator-related systemic symptoms, including recurrent anaphylaxis, abdominal pain, gastrointestinal distress, flushing, syncope, headache, hypotension, tachycardia or respiratory symptoms.[7] Furthermore, since mast cell mediators are known to play an important role in bone homeostasis, its alteration because of extensive release of mast cell mediators, often leads to musculoskeletal complaints such as bone pain, osteopenia/osteoporosis, pathological fractures and/or arthralgia.[7]

3.1.1.7. Immunophenotype of KIT-mutated mast cells

As previously referred, SCF induced activation of c-Kit plays an important role in a broad range of mast cell biological functions, including mast cell proliferation, survival, differentiation, migration, adhesion and activation, among others functions.[26,27] *KIT* mutation involving mast cells from patients with clonal mast cell disorders has a significant impact in most, if not all, these cell functions.[26] Such functional changes are the visible consequence of an altered expression of several molecules (Table 9) due to constitutive activation of c-Kit and its downstream pathways, as well as the altered communication with the cell microenvironment. Accordingly, increased mast cell secretion/degranulation in mastocytosis patients carrying the D816V mutation, is associated with up-regulation of membrane expression of activation (e.g. CD69[320]) and degranulation (e.g. CD63[321] and CD203c[115]) related molecules, as well as increased expression on BM mast cells of complement-associated proteins like CD35, CD59 and CD88.[124,321] Furthermore, the altered mast cell adhesion and migration functions that contribute to the abnormal accumulation of clonal cells in distinct tissues are partially due to an altered expression of adhesion molecules, such as expression of CD2 (LFA-2; absent in normal BM mast cells while detected in the majority of the patients),[113,322] up-regulation of CD33 (Siglec-3)[113] and

down-regulation of the CD29 integrin (integrin beta-1) in these patients *vs.* healthy subjects.[113]

Table 9. Qualitative and semiquantitative patterns of expression of individual markers on normal and clonal bone marrow mast cells. [Adapted from [110,113,122,124,125,322,323]]

Functional group of molecules	Antigen	CD code	Normal BM MC (% of positive cases)	Clonal BM MC (% of positive cases)
CYTOKINE RECEPTORS	IL-2Rα	CD25	-	++ (100%)
	c-Kit	CD117	+++ (100%)	++ (100%)
ADHESION-RELATED MOLECULES	LFA-2	CD2	-	+ (83%)
	Integrin alpha-L	CD11a	-/+ (20%)	-
	Integrin alpha-M	CD11b	-/+ (50%)	-/+ (50%)
	Integrin alpha-X	CD11c	-/+ (71%)	+/++ (100%)
	Integrin beta-2	CD18	-/+ (65%)	-/+ (44%)
	Siglec-2	CD22	-/+ (60%)	-/+ (60%)
	Integrin beta-1	CD29	++ (100%)	+/++ (100%)
	Siglec-3	CD33	++/+++ (100%)	+++ (100%)
	HPCA-1	CD34	-	-
	gpIIb/IIIa complex	CD41a	-	-/+ (45%)
	Platelet glycoprotein Ib alpha	CD42b	-	-/+ (45%)
	Pgp-1	CD44	++ (100%)	++ (100%)
	Integrin alpha-4	CD49d	+/++ (100%)	+/++ (80%)
	Integrin alpha-5	CD49e	+ (100%)	-/+ (30%)
	Integrin alpha-V	CD51	+ (100%)	-/+ (45%)
	ICAM-1	CD54	-/+ (75%)	++ (100%)
	Integrin beta-3	CD61	-/+ (66%)	-/+ (22%)
COMPLEMENT-RELATED PROTEINS	CR1	CD35	-	++ (100%)
	Membrane cofactor protein	CD46	+ [a]	+ [a]
	Complement decay-accelerating factor	CD55	++ (100%)	++ (100%)
	Membrane attack complex inhibition factor	CD59	++ (100%)	++/+++ (100%)
	C5aR	CD88	-	-/+ (50%)
IMMUNOGLOBULIN RECEPTORS	FcεRI	NA	++/+++ (100%)	++ (100%)
	FcγRIIIB	CD16	-	-
	FcεRII	CD23	-	-
	FcγRI	CD64	-	-
TETRASPANINS	CD9 antigen	CD9	+++ (100%)	+++ (100%)
	LAMP3	CD63	++ (100%)	++/+++ (100%)
TNF RECEPTOR FAMILY PROTEINS	CD30L receptor	CD30	-	-/+(38%) [b]
	CD40L receptor	CD40	-/+ (65%)	-/+ (65%)
ACTIVATION MARKERS	Early activation antigen CD69	CD69	+ (100%)	+/++ (100%)
	E-NPP3	CD203c	+ (100%)	+/++ (100%)
OTHERS MOLECULES	T-cell surface glycoprotein	CD4	-/+ (60%)	-/+ (60%)
	Aminopeptidase N	CD13	-/+ (33%)	-/+ (75%)
	ADP-ribosyl cyclase 1	CD38	-	-
	Leukosialin	CD43	+ (100%)	-/+ (80%)
	LCA	CD45	++ (100%)	++ (100%)
	Transferrin receptor protein 1	CD71	+ (100%)	-/+ (38%)
	Bcl-2	NA	+ (100%)	+/++(100%)
	Chymase	NA	-/+ [a]	-/+ [a]
	Tryptase	NA	++ (100%)	++ (100%)

a) Information regarding the frequency of positive cases is currently not available. b) Detected by immunohistochemistry;

NA: not applicable; HPCA-1- Hematopoietic progenitor cell antigen CD34; Pgp-1 - Phagocytic glycoprotein 1; ICAM-1 - Intercellular adhesion molecule 1; CR1 – complement receptor 1; LAMP3- Lysosomal-associated membrane protein 3; E-NPP3- Ectonucleotide pyrophosphatase/ phosphodiesterase family member 3; LCA, Leukocyte common antigen.

Interestingly, expression of CD25 (IL-2Rα) which is typically absent in normal/reactive BM mast cells, is only detected in mast cells from patients carrying the *KIT* D816V activating mutation, but not in reactive and clonal mast cells from many patients that lack this mutation.[113,257] Moreover, expression of other molecules, like CD30 (TNFRSF8), seems to be only detected in BM mast cells from a subset of D816V[+] patients with more aggressive forms of the disease;[323] other molecules, like the transferring receptor protein 1 (CD71) are not detected in neoplastic mast, cells while expressed by normal BM mast cells.[113]

3.2. Diagnostic criteria for mastocytosis

Mastocytosis defines a heterogeneous group of clonal disorders characterized by the accumulation of morphologically and/or immunophenotypically abnormal mast cells in one or multiple organs and tissues.[7] Depending on the disease distribution and its clinical manifestations, two major variants of the disease exist (cutaneous *vs.* systemic mastocytosis), which are further subdivided into different subtypes according to the type and sites of clonal mast cell involvement, laboratory findings and the degree of organ impairment.

Accordingly, cutaneous mastocytosis (CM) is more frequently diagnosed in children, based on the presence of skin involvement with typical histological infiltrates of mast cells in a multifocal or diffuse pattern, in the absence of extracutaneous disease; by contrast, systemic mastocytosis (SM) is most commonly seen in adults and it typically involves multiple organs, which almost always include the BM and often, also the skin.[7,324] The great majority (>90%) of adult cases with systemic mastocytosis carry activating mutations in the catalytic domain of *KIT* (most frequently the D816V *KIT* mutation). Such mutations lead to abnormal proliferation and migration processes, ultimately resulting in the expansion and accumulation of morphologically and/or immunophenotypically aberrant mast cells in distinct tissues. Since this represents the

most common form of the disease in adults, presence of multifocal dense aggregates of >15 (usually spindle-shaped), mast cells in tissue sections from biopsy specimens of bone marrow and/or other extracutaneous organs is considered by the World Health Organization (WHO), as the single major diagnostic criterion for systemic mastocytosis (Table 10).[7,325]

The BM is the most common site where diagnosis of SM is established, as it is almost always involved. However, diagnostic material can also be obtained from other sites, but histological criteria for the diagnosis of mastocytosis in extramedullary/extracutaneous organs, are less established.[324] In order to correctly identify mast cells in distinct tissues, specific immunohistochemical markers such as c-Kit and tryptase must be used.[325]

Table 10. WHO diagnostic criteria for systemic mastocytosis. [Modified from Horny et al.[7]]

Major criterion
• Multifocal, dense infiltrates of mast cells (≥15 mast cells in aggregates) detected in sections of bone marrow and/or other extracutaneous organ(s).
Minor criteria
1. In biopsy sections of bone marrow or other extracutaneous organs, >25% of the mast cells in the infiltrate have an atypical morphology (e.g. spindle shape) or >25% immature or atypical cells from all mast cells are found in a bone marrow aspirate smear.
2. Detection of an activating point mutation at codon 816 of *KIT* in bone marrow, blood or another extracutaneous organ.
3. Mast cells in bone marrow, blood or other extracutaneous organs aberrantly express CD2 and/or CD25.
4. Serum baseline total tryptase persistently exceed 20ng/mL.[a]
THE DIAGNOSIS OF SYSTEMIC MASTOCYTOSIS IS MET WHEN ONE MAJOR AND ONE MINOR, OR AT LEAST THREE MINOR CRITERIA, ARE PRESENT.

[a] *This criterion is not valid if there is an associated clonal myeloid disorder.*

Apart from this major diagnostic criterion, four additional minor criteria are proposed by the WHO for the diagnosis of SM. These include the presence of activating point mutations at codon 816 of *KIT* in mast cells from BM, blood or another extracutaneous organ. The D816V *KIT* mutation is present in most adult SM cases,

except for a small, clinically distinct, subgroup of patients.[7,257] However, detection of the *KIT* mutation should be performed with caution, specially because the majority of infiltrated samples have relatively low mast cell numbers (<0.01%). In such cases, usage of whole BM or BM mononuclear cells to obtain DNA or RNA is not recommended for *KIT* mutational analysis due to the limited sensitivity associated with many of the currently in-use molecular assays;[325] by contrast, *KIT* mutational analyses should be preferentially performed in highly-purified mast cells.[257] Different approaches have been applied to isolate mast cells for the study of *KIT* mutations, including microdissection of formalin-fixed paraffin-embedded BM and skin biopsies[270] and both immunomagnetic[326] and/or fluorescence-activated cell sorting (FACS) of mast cells from BM and peripheral blood samples.[257,314] Additionally, multiple molecular methods can be used to specifically detect the D816V *KIT* mutation in patients suspected of SM. Accordingly, mRNA/cDNA-based methods,[253,326] like reverse transcriptase (RT) polymerase chain reaction (PCR)-based assays, are more sensitive for the detection of the *KIT* mutation in samples that contain variable mixtures of mast cells and other cells, since mast cells, together with a small subset of basophils and NK cells, are the only mature hematopoietic cells expressing *KIT* mRNA.[314] Among the genomic DNA-based methods, direct sequencing is the simplest approach; however, this technique has very low sensitivity due to the fact that the D816V *KIT* mutation is usually heterozygous and, if the technique is not performed in highly-purified mast cells, mutated cells will be further diluted in a large number of cells carrying wild-type *KIT*.[314] Improved sensitivity has been reached by enhancing mutated allele frequencies either through the use of digestion steps with specific restriction endonucleases and PCR amplification[253,327] or by using specific probes that selectively block amplification of the wild-type sequence.[270] This later PNA-mediated wild-type blocking method has the advantage of allowing detection of the D816V *KIT* mutation, as well as other rare mutations involving the *KIT* TK2 regulatory domain at codons 815-819.[257,270] Other genomic DNA-based assays using allele-specific competitive blockers of the mutated

sequences and traditional[328,329] or quantitative PCR[330] assays, have also been shown to be associated with a good sensitivity (sensitivity threshold of between 1% and 0.03%, respectively).

A second minor criterion for the diagnosis of SM, takes advantage of the effects of constitutive activation of c-Kit in the cytoskeleton of clonal mast cells, resulting in abnormal morphological features: identification of >25% atypical mast cells in BM smears or biopsy sections (Table 10).[7,312,325] This requires detailed morphological analysis of bone marrow smears or biopsy sections using e.g. May-Grünwald-Giemsa, Wright-Giemsa and/or toluidine blue stainings.[312,325] However, atypical mast cell cytology is not specific for SM patients, as it can also be observed in disease conditions other than mastocytosis, such as a subset of hypereosinophilic syndromes (HES) with the *FIP1L1-PDGFRA* fusion gene.[331]

The third minor WHO diagnostic criterion for SM, relies on the presence of phenotypically aberrant CD25+ and/or CD2+ mast cells in the BM or other extracutaneous tissues. In contrast to normal mast cells, BM mast cells from mastocytosis patients show aberrantly high levels of expression of CD25,[110,257] except for a few cases in which no *KIT* mutation is found; in addition, CD2 is also aberrantly expressed by BM mast cells from most systemic mastocytosis patients. However, recent reports indicate that CD2 is less sensitive than CD25, as it is absent in some of those patients that show CD25+ mast cells, while it does not improve the sensitivity of CD25 for the diagnosis of the disease.[322] Thus, despite the WHO establishes the presence of CD25$^+$ and/or CD2$^+$ mast cells in BM, blood or other extracutaneous organs as a minor criteria for the diagnosis of systemic mastocytosis, at present it is considered that it would be more appropriate to use the criterion of CD25$^+$ mast cells, for the diagnosis of SM, independently of CD2 expression.[325] Although immunohistochemical and immuncytochemical detection of CD25 and CD2 is possible, flow cytometry appears to be more sensitive and it is currently considered the gold

standard for the evaluation of the expression of both molecules in BM mast cells from subjects suspected of systemic mastocytosis.[325]

The fourth minor criterion defined by the WHO for the diagnosis of SM includes a persistent increase in serum baseline tryptase levels over 20ng/mL.[7,252] It should be noted that such increased serum tryptase levels are not present at diagnosis in a substantial proportion of patients, especially among those cases with a low mast cell burden. In order to increase the sensitivity of serum baseline tryptase determination, it has been recently suggested that the cutoff used could be lowered.[332] Nevertheless, this feature is also not specific for SM, as increased serum tryptase may also be detected in other non-mastocytosis disorders, such as diffuse bone esclerosis,[333] other myeloid disorders like acute myeloid leukemia (AML), MDS and a subset of HES, which impairs the usage of increased serum tryptase for the diagnosis of SM in cases in which these hematopoietic and non-hematopoietic disorders coexist.[331,334-336]

According to the WHO, diagnosis of systemic mastocytosis is established when the major and at least one minor criteria are fulfilled or alternatively, when three or more of the four minor criteria are met, despite the absence of mast cell aggregates in the BM and other extracutaneous tissues (Table 10).[7] Recent studies suggest however, that additional criteria could be used for the diagnosis of this group of diseases. Accordingly, adult-onset patients with mastocytosis in the skin have a very high (>95%) probability of having systemic involvement, supporting the notion that mastocytosis in the skin has a high predictive value for systemic mastocytosis in adults.[337] In turn, those cases who have indolent forms of the disease lacking skin lesions, frequently present in males showing a mast cell activation syndrome, with acute episodes that are idiopathic or triggered by *Hymenoptera* sting.[325,338] These latter patients typically present very low BM mast cell burden associated in around one third of the cases (30%), with the absence of detectable BM mast cell aggregates (e.g. they lack the major WHO diagnostic criterion) and normal serum tryptase levels.[338] As a consequence, the diagnosis of SM is problematic and should be probably based on

careful evaluation of the three remaining minor criteria, based on sensitive flow cytometry and molecular approaches.[112,325]

Despite all the above cited limitations, at the present the WHO criteria remain the gold standard for the diagnosis of SM; however, one should consider that based on its limitations, a number of patients, particularly from some specific subtypes of systemic mastocytosis, may not fulfill the WHO diagnostic criteria,[8,337] therefore being misdiagnosed.[339] In order to improve the diagnosis of these small groups of SM patients, new diagnostic algorithms have been proposed by several groups including the Spanish Network on Mastocytosis (REMA). Accordingly, the REMA reported an increased sensitivity and specificity for the identification of SM patients, based on the presence of skin lesions and/or clonal BM mast cells, associated with an aberrant BM mast cell immunophenotype and/or presence of compact mast cell aggregates in bone marrow biopsy, independently of the serum tryptase levels.[337]

3.3. Diagnostic classification and distinct subtypes of mastocytosis

Patients with mastocytosis show an heterogeneous and diverse clinical presentation, with several different patterns which have led to the definition of distinct subtypes of the disease. In practice, classification of the distinct subtypes of mastocytosis is generally based on clinical presentation, pathological findings and the prognosis of the disease.[5]

3.3.1. Cutaneous mastocytosis (CM)

Since several subtypes of mastocytosis, including SM, frequently involve the skin, the diagnosis of CM requires the exclusion of systemic involvement, associated with the demonstration of typical clinical findings.[7] This non-systemic subtype of the disease frequently emerges in childhood and is associated with a favorable prognosis,

spontaneous regression frequently occurring around puberty in a significant proportion of cases.[324] Of note, adult-onset CM typically does not regress, and most of these patients have underlying systemic involvement.[340]

Recently, consensus criteria for the diagnosis of CM have been further refined, and three major variants are now recognized.[7] The most frequent form of CM is urticaria pigmentosa (UP)/maculopapular cutaneous mastocytosis (MPCM) occurring in both children and adults.[7] In children, the lesions tend to be larger and papular, whereas in adults the mast cell infiltrate is more subtle due to dissemination of the lesions which tend to be red or brown-red and macular or maculopapular.[7,324] Histologically, aggregates of spindle-shaped mast cells are typically seen, which fill the papillary dermis and extend as sheets and aggregates into the reticular dermis, often in perivascular and periadnexal positions; of note, adult UP tends to show fewer mast cells (Figure 4A and D). Diffuse CM is a distinct, less frequent form of CM, which
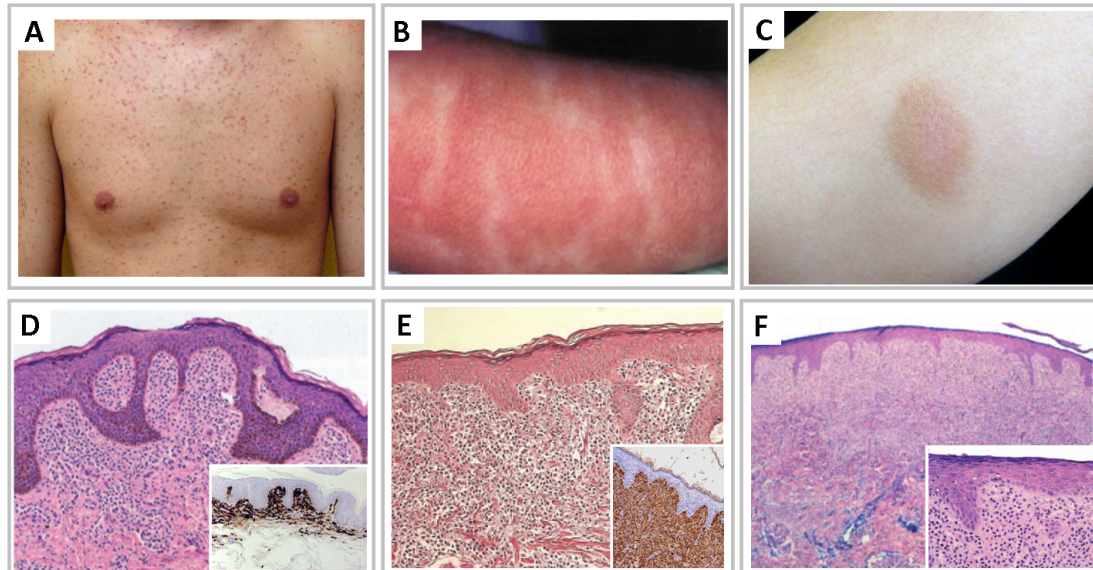

Figure 4. Dermoscopic (Panels A-C) and histopathological (hematoxylin and eosin staining in panels D through F, and tryptase immunohistochemical staining - insert in Panels D and E -) features of skin lesions from patients with mastocytosis. Illustrating urticaria pigmentosa lesions are shown in panels A and D, diffuse cutaneous mastocytosis lesions are shown in panels B and E, whereas panels C and F illustrate solitary mastocytoma lesions. [Modified om: Horny et al,[7] and Yanagihori, et al.[259]]

presents almost exclusively in childhood.[7] In these patients, the skin is typically thickened and may have a *peau d'orange* (orange peel) appearance, without distinct lesions; in addition, mast cells form a band-like infiltrate in the papillary and upper reticular dermis (Figure 4B and E).[7,324] The third variant of CM (e.g. skin mastocytoma) is almost exclusively seen of children and it typically occurs as a single lesion characterized by the presence of sheets of mature-appearing mast cells, with abundant cytoplasm lacking atypia, which densely infiltrate the papillary and reticular dermis and may extend into the deep dermis and subcutaneous adipose tissue (Figure 4C and F).[7,324]

3.3.2. Systemic mastocytosis (SM)

As previously stated, SM patients share some clinical, histopathological and biochemical features that allow for their differential diagnosis with cutaneous forms of the disease.[7] However, despite the fact that these patients typically show BM involvement, the clinical presentation of SM is diverse and distinct subtypes of the disease, with different biological, clinical and prognostic features, as well as distinct therapeutic requirements, are currently defined.[7] For the differential diagnosis of these subgroups of SM patients, a set of criteria, called "B"-findings (Borderline-Benign) and "C"-findings (Consider Cytoreduction or chemotherapy) are used, to assess the mast cell burden and the aggressiveness of the disease (Table 11).[7,256]

Apart from the WHO criteria, recent reports suggest that combined assessment of β2-microglobulin and LDH (lactate dehydrogenase) serum levels could contribute to a more objective differential diagnosis of systemic forms of the disease: increased β2-microglobulin and decreased LDH levels are closely associated with clinically aggressive forms of the disease, whereas coexistence of both parameters is only found in a minority of indolent SM cases.[337]

Table 11. WHO criteria for the differential diagnosis of distinct variants of systemic mastocytosis. [Modified from Alvarez-Twose et al.[314]]

"B" findings: *Associated with disease progression and a variable increase in mast cell burden*
1. Bone marrow biopsy with >30% infiltration by mast cells (focal, dense aggregates) by histology (and/or >1% by flow cytometry).   Serum tryptase levels >200ng/mL in the absence of diffuse bone sclerosis.   Development of diffuse bone sclerosis   Increased β2-microglobulin serum levels.   Hypercellular BM with loss of fat cells.
2. Discrete signs of dysmyelopoiesis in non-mast cell lineage cells without substantial cytopenias, and WHO criteria for MDS or MPN.
3. Organomegaly (palpable hepatomegaly, splenomegaly and/or lymphadenopathy >2cm on CT or US) without impaired organ function.
"C" findings: *Associated with impaired organ function due to mast cell infiltration (e.g. confirmed in a biopsy study)*
1. Cytopenia (neutrophil count <1.0 x 10^9/L, Hb<100g/L and/or platelets <100 x 10^9/L), due to BM dysfunction with no obvious other non-mast cell hematopoietic malignancy.
2. Hepatomegaly with impaired liver function and/or ascites.
3. Palpable splenomegaly with hypersplenism.
4. Malabsorption with hypoalbuminemia and weight loss.
5. Skeletal lesions: large-sized osteolytic lesions and/or pathologic fractures, associated with local mast cell infiltration (pathologic fractures associated with osteoporosis should not be considered a "C"-finding)
6. Life-threatening organopathy in other organ systems caused by infiltration of the tissue by neoplastic mast cells.

CT, computerized tomography; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; US, ultrasound; WHO, World Health Organization; Hb, hemoglobin, BM, bone marrow.

3.3.2.1. Indolent Systemic Mastocytosis (ISM)

Indolent SM (ISM) is the most common subtype of systemic mastocytosis, with a frequency that ranges from 46% of SM cases[327] to 79% of all mastocytosis,[325] in two distinct studies. This variant of SM is defined by the presence of morphologically atypical and CD25-positive mast cells that carry *KIT* mutation, in association with multifocal mast cell aggregates in a significant but variable percentage of cases (usually >70%) and increased serum tryptase levels above 11.5ng/mL (>95% of cases) or 20ng/mL (>75% of cases).[325] Additionally, ISM patients typically lack "B" and "C" findings and do not show an associated clonal hematological non-mast cell lineage

disease (AHNMD); despite this, around one fifth to one third of the cases that show skin lesions (20-30%) display multilineage (myeloid or even myeloid plus lymphoid) involvement of hematopoiesis by the *KIT* mutation, while this is rarely detected in cases that lack skin lesions.[7,257] The presence of skin lesions is a common feature to the great majority of ISM patients; however, around one third of the cases lack such lesions.[325] This later subgroup of ISM includes ISM patients recognized by the WHO as isolated bone marrow mastocytosis (BMM).[7] However, a series of reports[338,341,342] suggest that the great majority of them could in fact represent a distinct subtype of ISM (ISM without skin lesion or ISMs-) as they show male prevalence, a higher age of onset of the disease and lower serum baseline tryptase, compared to ISM patients with skin lesions (ISMs+); in addition, ISMs- cases frequently show associated anaphylaxis and/or severe mast cell mediator related symptoms, triggered by a *Hymenoptera* sting, and they typically carry exon 17 *KIT* mutations restricted to the aberrant mast cell population.[257,338,341] Therefore, ISMs- patients clearly differ from BMM, which represents an exceptional subcategory of ISM patients in the absence of skin lesions and mast cell-mediator symptoms, which is usually diagnosed during a BM study for pathological conditions other than mastocytosis.[7,8,343]

Additionally, the WHO recognizes another subgroup of ISM patients, namely smoldering SM (SSM). SSM is characterized by a high mast cell burden associated with the presence of two or more "B" findings.[7,324] This particular subgroup of patients has been described to be significantly older and to display a higher incidence of constitutional symptoms (fatigue, weight loss, fever, diaphoresis) and anemia, together with a shorter survival, compared to other ISM patients.[324,344]

3.3.2.2.Well-differentitated Systemic Mastocytosis (WDSM)

Well-differentiated SM (WDSM) is a rare category of SM (≈5% of all mastocytosis patients),[325] characterized by frequent pediatric onset, nodular skin

lesions that usually involve a high percentage of the body surface area, and that mainly localize in the trunk and neck, with severe mast cell mediator related symptoms at onset, and persistence of the disease in adulthood in only a small percentage of cases.[325] Interestingly, around 40% of these cases[345] do not fulfill the WHO criteria for SM, since their BM mast cells show a normal-appearing round-shaped morphology with increased size and a fully granulated cytoplasm, in the absence of morphological atypia,[325] they frequently lack CD25 and CD2 expression,[265,346] and *KIT* mutations in exon 17 cannot be detected in around 85% of the cases.[257] In these latter cases, lacking *KIT* mutations, assessment of mast cell clonality should be performed whenever possible, using other techniques like those investigating the pattern of inactivation of polymorphic genes coded in human chromosome X (e.g. PCR-based human androgen receptor allele – HUMARA- assay) in women.[314]

3.3.2.3. Aggressive Systemic Mastocytosis (ASM)

Aggressive SM (ASM) represents a minoritary group of SM patients ($\approx$12% of all SM patients)[327] who may or may not have skin lesions.[325] These patients typically fulfill all diagnostic criteria for SM, with serum tryptase usually higher than 200ng/mL, severe mast cell morphological atypia and *KIT* mutation present in other myeloid or even myeloid plus lymphoid hematopoietic lineages.[257] These patients typically display hepatosplenomegaly and/or lymphadenopathy, fulfilling at least one "C"-finding, and eosinophilia (without *PDGRFA* translocations) is also a frequent coexisting finding.[325] BM studies usually reveal a high but variable degree of mast cell infiltration, sometimes with features of myelodysplasia and/or myeloproliferation, without fulfilling the WHO criteria for another clonal hematologic non-mast cell lineage disease (AHNMD).[7] Additionally, extensive bone involvement is also frequently found in these patients (e.g. fibrosis, diffuse bone sclerosis, or a mixed sclerotic and lytic pattern).[325]

3.3.2.4. Mast cell leukemia (MCL)

Mast cell leukemia (MCL) is a very infrequent (1-2% of cases) and aggressive form of mastocytosis, defined by an increase in BM mast cells equal or greater than 20% of all nucleated cells in aspirated BM smears; BM biopsies typically show diffuse, usually compact, infiltration by highly atypical immature mast cells (promastocytes or even, less frequently, metachromatic blasts) with marked reduction of fat cells and normal hematopoietic precursors.[7,325] These patients typically lack skin lesions and in typical cases, mast cells account for ≥10% of the leukocytes in peripheral blood, except for an aleukemic variant with <10% peripheral blood infiltration.[7,324] Serum tryptase is almost always elevated and *KIT* mutations are found in most patients.[257,324] "C"-findings are frequently associated, as most cases show rapidly progressing organopathy (involvement of liver, BM and other organs), associated with rapid clinical deterioration.[347] In most patients, mast cells aberrantly express CD2 and/or CD25, and CD30 expression can also be found in a proportion of the mast cells.[7,323]

3.3.2.5. Systemic mastocytosis associated with other clonal hematologic non-mast cell lineage disease (SM-AHNMD)

Systemic mastocytosis associated with other clonal hematologic non-mast cell lineage disease (SM-AHNMD) is a unique and heterogeneous subgroup of SM that requires separate classification of both neoplasms.[7,325] The SM component may correspond to an indolent or aggressive SM and should be classified as such, based on the WHO criteria.[7,325] The associated hematological neoplasm may be of any type, but myeloid neoplasms (e.g. AML, MDS or MPN) are more frequent than chronic lymphoid disorders.[325] The cytologic, immunophenotypic and molecular features of BM mast cells from these cases typically resemble those of the corresponding subtype of SM

(e.g. ISM or ASM).[325] However, in some cases the *KIT* mutation can be found in both the SM and the myeloid (non-mast cell) components, in contrast to lymphoproliferative neoplasms, in which the *KIT* mutation is typically absent in the malignant lymphoid component.[348] However, the clinical impact of the presence of a *KIT* mutation in the AHNMD component, still remains unknown and deserves further investigations.

3.3.3. Other forms of mastocytosis

3.3.3.1. Mast cell sarcoma

Mast cell sarcoma is an extremely rare pathological condition characterized by a local and destructive growth of highly atypical mast cells, which can only be correctly identified upon immunohistochemical staining with mast cell specific markers (e.g. CD117, tryptase or CD25), in the absence of systemic involvement.[7] Additionally, diagnosis can also be achieved by multiparameter flow cytometry immunophenotyping, which allows for the detection of aberrant immunophenotypic patterns.[325] Only few cases have been reported and the sites of involvement include the larynx, colon, meninges, bone and skin.[324,349-352] Furthermore, some patients may develop a terminal leukemic phase which is indistinguishable from MCL.[7]

3.3.3.2. Extracutaneous mastocytoma

Extracutaneous mastocytoma is an extremely rare disorder, consisting of a localized benign tumor resulting from an accumulation of mature-appearing granulated, strongly metachromatic mast cells, with no significant cytological atypia.[7] Most of the cases occur in the lung without skin lesions or evidence of systemic disease; in contrast to mast cell sarcoma, mastocytoma generally does not progress to systemic disease or show leukemic involvement.[324,353,354]

3.4. Prognosis and predictive factors in mastocytosis patients

As referred above, CM in children usually has a favorable outcome and it may even regress spontaneously before or during puberty.[7] Nonetheless, even though the disease may regress, some of these patients may show clinical severe, even life-threatening mast cell activation events, requiring emergency therapy and hospitalization, as a result of massive mast cell degranulation.[355] Accordingly, a recent study on a large cohort of children with CM (n=111), revealed that serum baseline tryptase values correlated with both the extent of skin involvement and the clinical severity of the disease at presentation, serum baseline tryptase levels >16 and >31ng/mL being associated with requirement for hospitalization and need to be treated in an Intensive Care Unit (ICU), respectively, therefore allowing for the identification of those patients at risk of severe mastocytosis-related anaphylaxis.[355]

Regarding SM, currently there is no cure for the disease, and the prognosis depends on both the disease category and its biochemical and molecular features.[327,356] Thus, distinct subtypes of the disease are associated with drastically distinct prognoses, ranging from a dismal prognosis, with a median survival of only 2 months in MCL, to a survival similar to an age-matched population not carrying a clonal mast cell disorder, in most ISM patients.[327] ASM and SM-AHNMD patients show a significantly reduced survival (overall median survival of 41 and 24 months, respectively), compared to indolent forms of the disease.[327] However, in those patients carrying an additional non-mast cell related disorder, prognosis has been reported to be highly dependent on the non-SM disease,[7] as SM-MPN patients have been described to have a significantly longer median survival (31 months) compared to patients with an associated chronic myelomonocytic leukemia (CMML) (median overall survival of 15 months), SM-MDS (median of 13 months) or cases with an associated acute leukemia (median of 11 months).[327] Additionally, a significant and independent association between survival and several predictive factors, including the WHO

subtype, advanced age, history of weight loss, anemia, thrombocytopenia, hypoalbuminemia and excess of BM blasts, has also been reported.[327] Interestingly, despite ISM patients typically show a long life expectancy, disease progression to more aggressive forms of the disease (e.g. ASM, MDS or AML) has been described to occur in ISMs+ patients which show extensive involvement of hematopoiesis (myeloid and/or myeloid plus lymphoid involvement) by the *KIT* D816V mutation.[356] Multilineage involvement of hematopoiesis by the *KIT* mutation is typically associated with the poor-prognosis variants of the disease (e.g. ASM, SM-AHNMD and MCL), but also detected in around 20% of ISMs+ cases.[257] Of note, apart from the presence of multilineage D816V *KIT* mutation, the detection of increased β2-microglobulin levels at diagnosis has also been identified as an independent predictor for transformation of ISM into more aggressive forms of the disease.[356] Additionally, recent observations based on a series of 74 patients with ISM who have been prospectively followed for a median of close to 8 years have shown that those patients who experienced progressively increased serum baseline tryptase levels throughout the study are more likely to show disease progression, whereas in those with stable serum tryptase levels, the disease maintains as an indolent condition (Matito A, unpublished data). The possibility of monitoring those ISM patients with high probability of progression to more aggressive forms of the disease, based on serum baseline tryptase levels could potentially allow for the detection of early stages of progression, leading to the suggestion of a less "static" classification of those ISM patients who typically carry multilineage myeloid and/or myeloid plus lymphoid D816V *KIT* mutation, who start to show signs of progression (e.g. "B" findings, including progressive hepatomegaly, usually together with lymph node enlargement and increased serum baseline tryptase levels). Interestingly, such variant of "in progression" ISM cases defined by the REMA, do not overlap with smoldering mastocytosis, which is defined more stringently.[314,325]

Regarding WDSM patients, the probability of progression to aggressive SM remains currently unknown. However, in a series of 17 cases, progression to ASM was detected in 2 of them, 21 and 71 years after the onset of the disease, respectively.[325]

3.5. Treatment of mastocytosis

Currently, no curative therapies exist for mastocytosis. Despite this, multiple different treatment strategies are used for the management of mastocytosis patients; such distinct treatment modalities should be carefully selected for individual cases based on the heterogeneous and highly variable clinico-biological features and prognosis of the disease.[357] Basic management principles include careful counseling of patients and care providers, avoidance of factors triggering mast cell mediator release, treatment of acute and chronic mediator-release symptoms and, if indicated, cytoreductive therapy in order to treat extensive mast cell organ infiltration, typically observed in aggressive cases.[358]

Avoidance of known and potential triggers for mast cell-activation associated symptoms of mastocytosis is mandatory, because risk for systemic hypotension due to massive mast cell mediator release, exists.[358] Therefore, stimuli known to induce mast cell degranulation (e.g. some drugs used in anesthetic procedures, iodinated radiocontrast dyes, opioid analgesics and insect sting) should be avoided, whenever possible.[357] In turn, in case of repeated life-threatening anaphylactic and anaphylactoid episodes, immediate self-administration of epinephrine is frequently recommended.[359]

The primary goal of the treatment of all clinical forms of mastocytosis is to inhibit mast cell mediator release and block their effects, in order to control the symptoms and signs of the disease due to mast cell activation.[358] Accordingly, symptoms associated with histamine release (e.g. flushing, pruritus and tachycardia), one of the major mast cell mediators, are typically treated with inhibitors of H1 and H2 histamine receptors, but total relief of the symptoms is rarely achieved.[344] Options for the

treatment of gastro-intestinal symptoms (e.g. diarrhea, nausea, vomiting or malabsortion) include proton pump inhibitors, that diminish diarrhea in addition to controlling gastric acid hypersectretion,[358] and oral sodium cromoglicate, an anti-inflammatory mast cell stabilizer with the ability to modulate sensory nerve function,[360] which has proven to be effective in controlling diarrhea, abdominal pain, nausea, and vomiting.[358] Additionally, topical sodium cromoglicate is also useful for the treatment of pruritus.[361] Similarly, glucocorticoids have also been shown to be beneficial in treating skin symptoms (e.g. urticaria pigmentosa) as well as complications of severe mastocytosis like malabsorption, hepatic fibrosis and ascites.[357] For the treatment of cutaneous symptoms (e.g. pruritus, whealing, and flare reactions), oral methoxypsoralen plus UVA (PUVA) radiation can also be administered; however, skin lesions often return within 3 to 6 months after treatment discontinuation.[358] Due to BM infiltration by mast cells, bone alterations (osteoporosis, osteopenia or lytic bone lesions) are frequently observed in a subset of patients, due to the release of mast cell mediators (e.g. histamine or tryptase) or mast cell mediators involved in the promotion of inflammatory responses (e.g. cytokines and leukotrienes), with both direct and indirect effects on bone cells. Recommended treatment for osteopenia and osteoporosis includes usage of bisphosphonates together with calcium and vitamin D supplementation.[357]

Cytorreductive therapy is only recommended for the treatment of aggressive, advanced, or refractory disease, due to the highly mutagenic potential of these drugs and their toxic side-effects.[276,357] Standard therapy for patients with aggressive forms of the disease has not been defined so far; however, several cytotoxic agents have shown a variable degree of efficiency in the treatment of these patients.[5] Among those agents, interferon-α (IFN-α), a glycoprotein with immunomodulatory and antiproliferative effects (often administered in combination with glucocorticoids)[362] has been associated with variable success in the treatment of ASM.[363] IFN-α treatment is frequently (up to 50% of the cases) associated with important side-effects, including

flu-like symptoms, bone pain, fever, cytopenias, depression and hypothyroidism.[276] Furthermore, a significant proportion of the patients will relapse shortly after IFN-α treatment is discontinued.[364] Some studies have stated that cladribine (2-CdA) may induce clinical responses in patients with more aggressive forms of mastocytosis, even in advanced IFN-α resistant disease.[365-368] However, not all patients respond to the treatment, specially when the disease progresses rapidly; in addition, 2-CdA may induce pancytopenia and immunosuppression.[5,359]

More recently, targeted therapies have been proposed to the treatment of ASM and MCL patients. Nevertheless, usage of such drugs (e.g. imatinib, an ATP-competitive kinase inhibitor) mandatorily requires definition of the exact molecular defects that underlie mastocytosis and the potential targets expressed by neoplastic cells.[359] As an example, sustained responses to imatinib have been achieved in cases carrying wild-type *KIT* or transmembrane/juxtamembrane mutations (F522C and K509I, respectively),[264,265,369] whereas other mutations, in particular the D816V mutation detected in >90% of SM patients, lead to resistance to imatinib.[370] Therefore, only those rare cases lacking *KIT* mutations (e.g. WDSM) or carrying a sensitive mutation may be appropriate candidates for imatinib therapy.[276] Other, investigational targeted drugs have been described to either lack (e.g. masatinib mesilate – AB1010[371]) or show modest activity in *KIT* D816V$^+$ SM (e.g. dasatinib[372]); those few drugs which have shown some activity (e.g. midustaurin – PKC412) require demonstration of their potential advantage over IFN-α or 2-CdA therapy, which are currently considered first line treatment options for the more aggressive forms of the disease.[276] The potential benefit in the treatment of SM of other compounds which are not classified as primary tyrosine kinase inhibitors, but that also inhibit c-Kit activity or c-Kit mediated mast cell activation (e.g. the rapamycin mTOR inhibitor, or the IMD-0354 NFκ-B inhibitor), deserves further *in vivo* investigations.[373]

Non-pharmacological therapeutic approaches for mastocytosis include splenectomy in patients with hypersplenism, to decrease mast cell burden and

improve cytopenias. Nevertheless, this is a high-risk procedure when applied to patients with aggressive forms of the disease and post-splenectomy survival has been reported to increase only by an average of 12 months.[357] On the other hand, current experience with BM transplantation in SM patients remains limited, with both favorable engraftment and prolongation of life, but an overall poor outcome due to graft-versus-host-disease and death. [357]

New potential therapeutic approaches include antibody-based constructs and drugs directed at surface molecules expressed on mast cells. Accordingly, mast cells express a wide array of proteins on their surface membrane (e.g. CD22, CD33 and CD52), for which antibody-based therapeutic agents are already available (e.g. Epratuzumab, Mylotarg and Alemtuzumab, respectively).[125,374] In addition, fusion protein-toxins which combine a targeting ligand (e.g. IL-2) with a bacterial toxin (e.g. $DAB_{486}IL$-2) have also been built and described to target some of the surface molecules expressed by clonal mast cell (e.g. the CD25 IL-2 receptor α chain).[125,375]

Chapter 2 |

HYPOTHESIS AND OBJECTIVES

Mastocytosis comprises a heterogeneous group of disorders which are mainly characterized by an abnormal accumulation of clonal mast cells in one or more organs systems. Despite mastocytosis is considered a unique disease, several distinct diagnostic entities are currently recognized by the WHO. These entities range from variants of the disease with tissue involvement restricted to the skin, with typical childhood onset and frequent regression at puberty, to chronic systemic disorders more frequently observed in adults, with either an indolent (e.g. ISM) or a more aggressive (e.g. ASM, MCL) behavior, associated with a distinct prognosis and different therapeutic requirements. In addition, recent reports have provided compelling evidence for the existence of two additional subvariants of indolent SM (e.g. WDSM and ISMs-) to those defined by the WHO, such new subvariants displaying unique clinco-biological features both at diagnosis and during follow-up.

Although patients carrying distinct variants of SM exhibit very different clinical features, presence of the *KIT* D816V mutation is detected in >90% of the cases, except among WDSM patients. The D816V *KIT* mutation induces constitutive (SCF-independent) activation of c-Kit, which would lead to an altered balance between mast cell proliferation and survival, in association with unique changes in the morphological (e.g. spindle-shaped mast cells), immunophenotypical (e.g. aberrant expression of CD25) and functional (e.g. enhanced mediator release) properties of clonal mast cells. However, the presence of this particular mutation *per se* does not explain the clinical, biological and histopathological heterogeneity observed among the distinct subtypes of SM. In this regard, additional molecular alterations have been reported among some ASM (e.g. *NRAS* and *TET2* mutations) as well as in ISM patients with higher risk of progression to more aggressive forms of the disease (e.g. ISM patients showing multilineage involvement of BM hematopoietic cells by the *KIT* D816V mutation); however, the specific molecular mechanisms, biological functions and signaling

pathways which are associated with each of the distinct variants of the disease, specially those carrying the same D816V *KIT* mutation, remain largely unknown.

In this regard, it should be noted that at present very limited knowledge exists about the GEP of BM mast cells from patients with mastocytosis. In fact, three studies have reported so far GEP of mastocytosis patients using cDNA-oligonucleotide arrays, but none of them has specifically investigated the gene expression profile of (highly-purified) BM mast cells. In fact, in these studies either BM mononuclear cells or whole peripheral blood samples were used for such purposes, both types of samples containing a complex admixture of various different cell populations, typically associated with a variably low degree of infiltration by mast cells; altogether, this has led to complex and biased results which are difficult to interpret.

Despite the lack of information about the GEP of mast cells in mastocytosis, multiparameter flow cytometry (MFC) assessment of protein expression profiles in single cells, has been shown to contribute to the diagnosis of SM through the identification of aberrant expression of several proteins (e.g. CD25 and/or CD2) on BM mast cells. However, current knowledge about the immunophenotypic features of BM mast cells from patients with distinct subtypes of SM also remains rather limited. This is mostly due to the fact that previous reports which focused on the analysis of the immunophenotype of mast cells in SM have not discriminated among the different subtypes of the disease, they have just studied a relatively small number of molecules in restricted cohorts of patients or they were restricted to a specific diagnostic subgroup of the disease, and no systematic study in a large and representative cohort of patients, which included all distinct variants of the disease, has been performed so far. On top of this, no study has investigated the potential association between the immunophenotypic profile of mast cells and the clinical and biological features of ISM patients, particularly among those cases who show disease progression and a worse clinical outcome (e.g. multilineage involvement of BM cells by the D816V *KIT* mutation). This would be particularly relevant if we consider that currently, determination of the

degree of BM involvement by the *KIT* mutation requires complex genetic analyses of highly purified BM cell populations, which are not easily available at routine diagnostic laboratories, whereas MFC is broadly distributed.

Based on the above background, the **general objective** of this study aimed at investigating the gene and protein expression profiles of BM mast cells from distinct variants of SM and determining their potential utility in the diagnostic and prognostic work-up of the disease. In order to accomplish this goal, we addressed the following three **specific objectives**:

- To determine the specific immunophenotypic characteristics of BM mast cells from patients diagnosed with different subtypes of SM;

- To evaluate the potential association between the immunophenotypic features of BM mast cells from ISM patients and the degree of involvement of the distinct compartments of hematopoietic BM cells by the *KIT* D816V mutation, and determine the utility of the protein expression profile of BM mast cells as a potential surrogate marker for multilineage disease, and;

- To investigate the GEP of BM mast cells from SM patients carrying the D816V *KIT* mutation and analyze its relationship with the distinct variants of the disease.

Chapter 3 |

MATERIAL, METHODS AND RESULTS

In the following section of this doctoral thesis a description of the patients from whom samples were obtained and studied, the materials and methods used, as well as the results obtained, is given for each of the proposed objectives, through those manuscripts which have been published or which acceptance for publication is pending, that were written as a result of the work performed. Accordingly, the following chapters are included in this section:

Regarding the **first objective of the study**, focused on the determination of the immunophenotypic profile of BM mast cells from distinct subtypes of SM, the following work is presented:

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

Teodosio C, García-Montero AC, Jara-Acevedo M, Sánchez-Muñoz L, Alvarez-Twose I, Núñez R, Schwartz LB, Walls AF, Escribano L, Orfao A.

Journal of Allergy and Clinical Immunology 2010; 125(3):719-26.

The work related to the **second aim of the study** is described in the following chapter, addressing the potential association between the immunophenotypic profile of BM mast cells in ISM and the degree of involvement of BM hematopoiesis by D816V *KIT* mutation, and its potential utility for the diagnostic screening of multilineage disease:

3.2. **An immature immunophenotype of bone marrow mast cells predicts for multilineage D816V *KIT* mutation in systemic mastocytosis.**

Teodosio C, García-Montero AC, Jara-Acevedo M, Alvarez-Twose I, Sánchez-Muñoz L, Almeida J, Morgado JM, Matito A, Escribano L, Orfao A.

Leukemia 2012; 26(5):951-8.

Regarding the **third objective**, focused on the description of the GEP of (highly purified) BM mast cells from distinct subtypes of SM carrying the D816V *KIT* mutation, the following work is presented:

3.3. **Gene expression profile of highly-purified bone marrow mast cells in systemic mastocytosis.**

Teodosio C, García-Montero AC, Jara-Acevedo M, Sánchez-Muñoz L, Pedreira C, Alvarez-Twose I, Matarraz S, Morgado JM, Barcena P, Matito A, Escribano L, Orfao A.

Journal of Allergy and Clinical Immunology (Accepted pending revision - Manuscript N. JACI-D-12-00708)

3.1. **MAST CELLS FROM DIFFERENT MOLECULAR AND PROGNOSTIC SUBTYPES OF**

SYSTEMIC MASTOCYTOSIS DISPLAY DISTINCT IMMUNOPHENOTYPES

Journal of Allergy and Clinical Immunology 2010; 125(3):719-26

Teodosio C. performed the MFC experiments, analyzed/interpreted the data, made the figures and wrote the paper.

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 immunophenotypical features, and aberrant expression of CD25 and/or CD2[7] 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 CD63[9] and CD69[10] activation molecules, CD58—a ligand for the CD2 protein[11]—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 β1-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
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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 SM[27] 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 immunophenotypical 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 (≥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 >mean ± 1 SD and a score of 0 or 1 was given when the MFI of individual markers was ≤ mean ± 2 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 >mean ± 2 SD but < mean ± 3 SD and when it was >mean ± 3 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 ± 1 SD) versus normal BM (1.7% ± 6.8% vs 0.07% ± 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 carboxy-peptidase, 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 ≤ .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
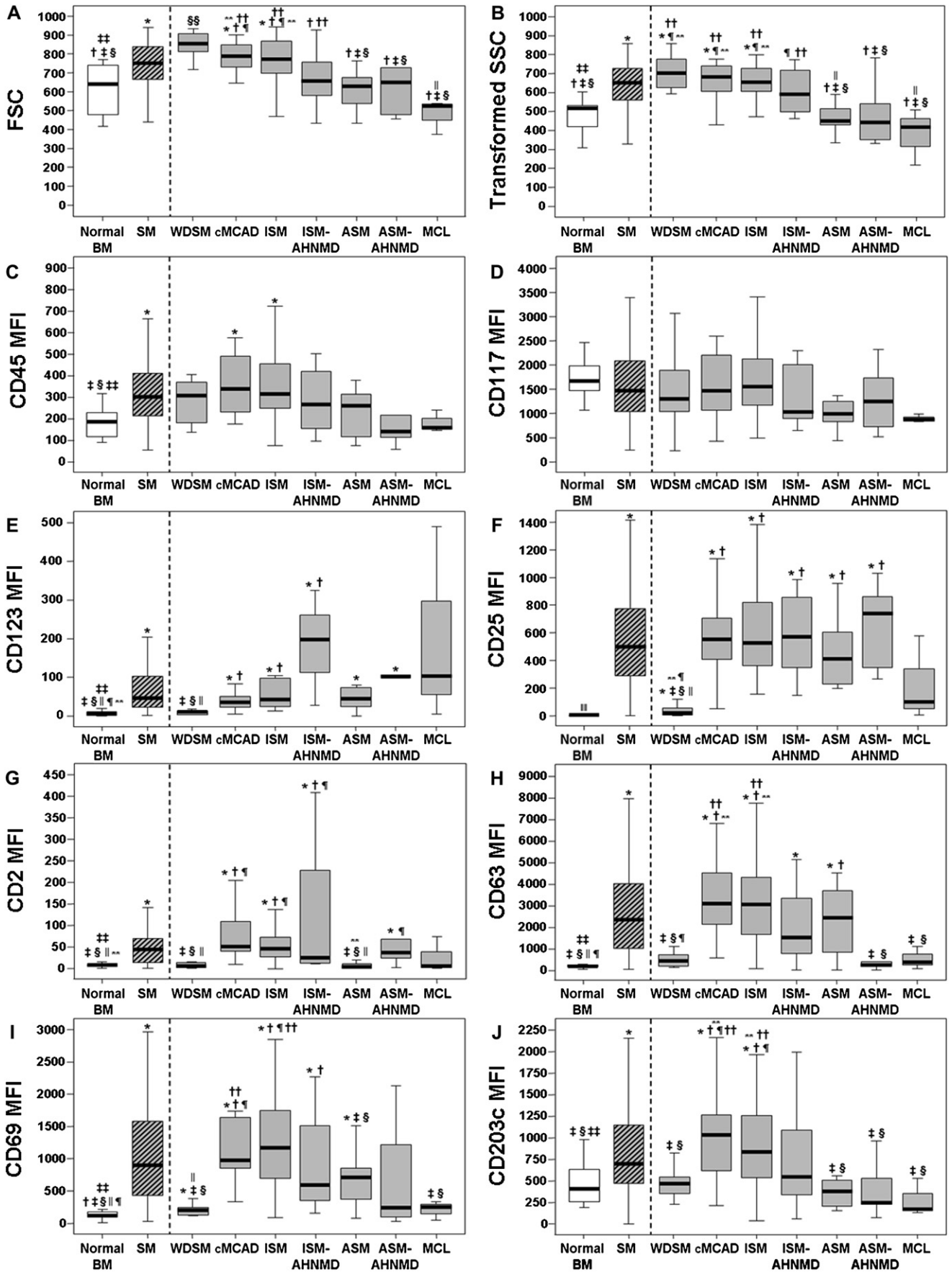
($P = .04$), CD16 ($P = .03$), and CyG5, which showed a higher and more heterogeneous expression in ASM-AHNMD versus ASM (Figs 1-3).

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 VI815-816 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 immunophenotypical 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 BMMCs[29] 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 INFγ—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 immunophenotypical 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 cases[16] 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 MCs[36] 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

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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.
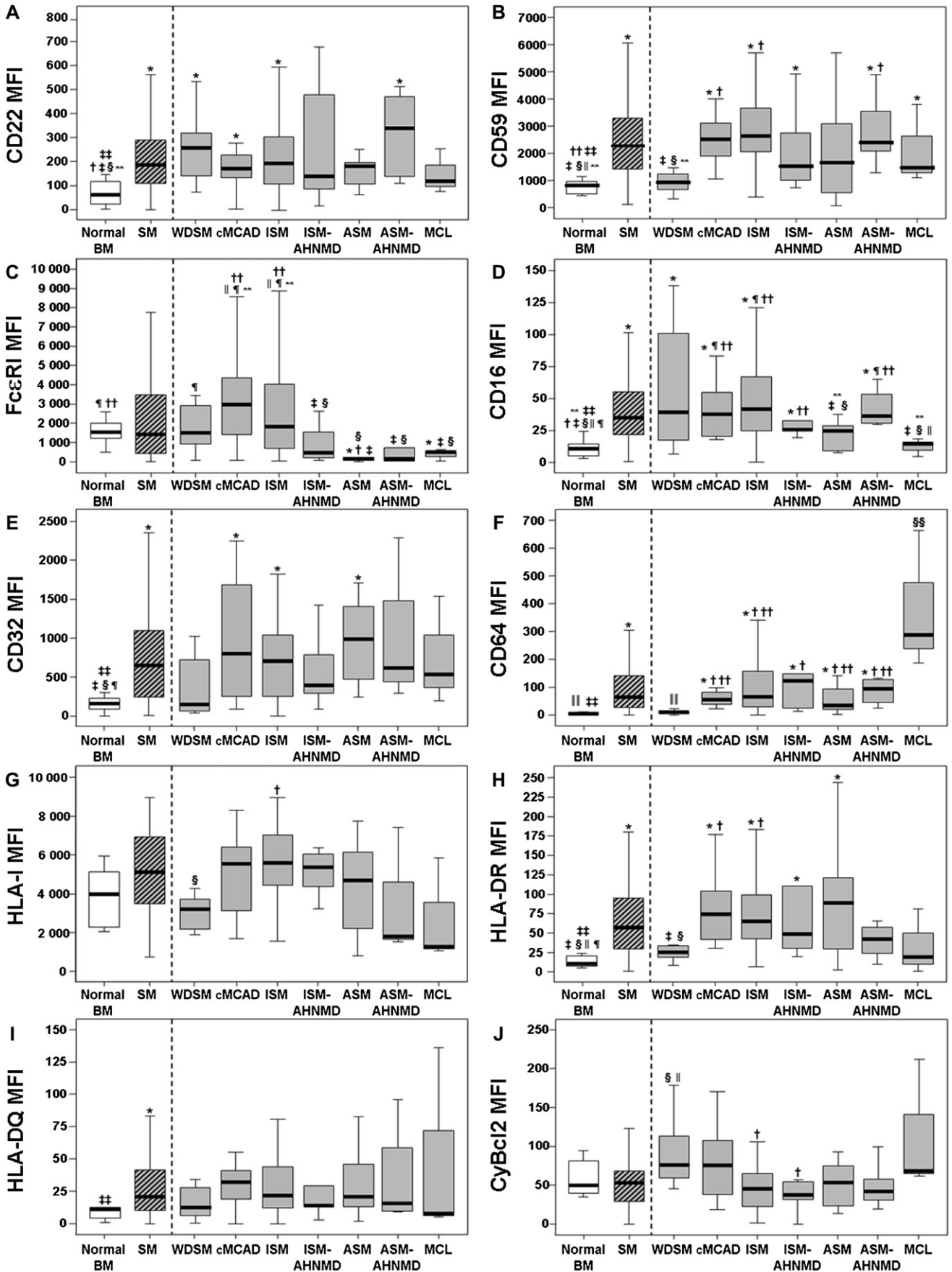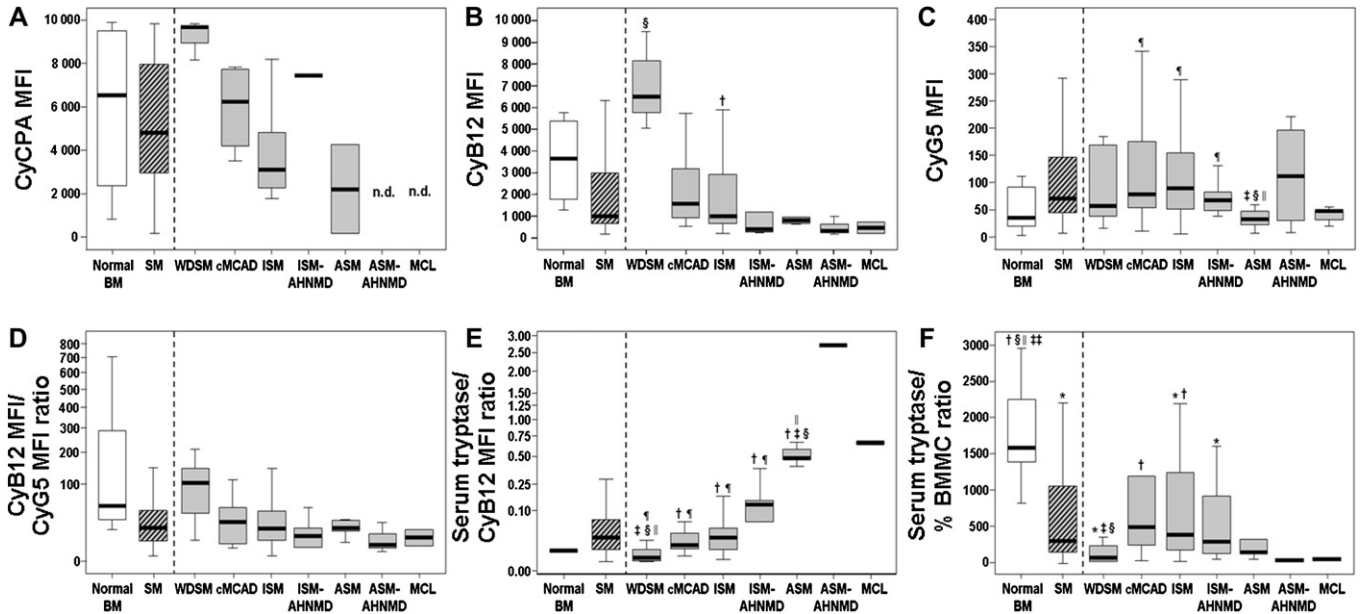
FIG 3. Serum tryptase levels (μg/L) and cytoplasmic expression of MC enzymes (mean fluorescence intensity *[MFI]*) per BMMC (CD117^{hi}/CD45^{+} with intermediate-to-high scatter cells) from patients with SM versus normal/reactive BMMCs. **A-C,** Expression of cytoplasmic carboxypeptidase A (CPA), total *(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 ‡‡SM. *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, FcεRI, 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)**, 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.

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RESULTS

Association between the immunophenotype of clonal BMMCs and other disease features

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

| | | Diagnostic subtype of SM | | | | | | |
| | | Good-prognosis categories | | | Poor-prognosis categories | | | |
	Controls (n = 92)	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[c,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 *KIT*[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)[e]
-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 [a]number 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 [b]controls, [c]WDSM, [d]cMCAD, [e]ISM, [f]ASM, [g]ISM-AHNMD, [h]ASM-AHNMD, [i]MCL, [j]all other groups, and all other groups except: [k]controls, [l]WDSM and ISM, [m]WDSM and cMCAD, and [n]WDSM and ASM-AHNMD.

[o]Not all patients had mutational analysis, and in some cases only purified MCs (but not other highly purified BM cell fractions) were analyzed.

[p]Among patients with *KIT* mutation, 5 had a mutation distinct from D816V (D816Y, I817V, V819Y, and V560G mutations and a VI815-816 insertion were detected each in 1 case).

TABLE E2. Antibodies used for the immunophenotypic analysis of BMMCs

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 *c*arboxypeptidase A; *N,* expression pattern similar to control BMMC; ↑, increased expression versus control BMMC; ↓, decreased expression versus control.

3.2. **AN IMMATURE IMMUNOPHENOTYPE OF BONE MARROW MAST CELLS PREDICTS FOR MULTILINEAGE D816V *KIT* MUTATION IN SYSTEMIC MASTOCYTOSIS**

Teodosio C. performed the MFC experiments, analyzed/interpreted the data, made the figures and wrote the paper.

npg

ORIGINAL ARTICLE

An immature immunophenotype of bone marrow mast cells predicts for multilineage D816V *KIT* mutation in systemic mastocytosis

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D816V *KIT* mutation of bone marrow (BM) mast cells (MC) is a common feature to systemic mastocytosis (SM) patients. Nevertheless, occurrence of the *KIT* mutation in BM cell compartments other than MC is associated with progression to more aggressive forms of the disease and poor outcome in indolent SM (ISM). Here, we assessed the potential association between the immunophenotype of MC and multilineage *KIT* mutation in the BM of SM patients through the investigation of the flow cytometric protein expression profile (PEP) of bone marrow mast cells (BMMC) from 70 control individuals and 206 SM patients, classified according to the WHO (World Health Organization), and the degree of involvement of BM hematopoiesis by the D816V *KIT* mutation; additionally, we developed a score-based class prediction algorithm for the detection of SM cases with multilineage mutation. Our results show that aberrant expression of CD25 with a FcεRI^{lo}, FSC^{lo}, SSC^{lo} and CD45^{lo} immature phenotype of BMMC, in the absence of coexisting normal MC in the BM, was associated with multilineage involvement by the D816V *KIT* mutation, regardless of the diagnostic subtype of the disease (for example, indolent vs aggressive SM), which supports the utility of the immunophenotype of BMMC as a surrogate marker to screen for multilineage *KIT* mutation in ISM.

INTRODUCTION

Mastocytosis is a heterogeneous group of disorders characterized by an abnormal growth and accumulation of neoplastic mast cells (MC) in one or more organ systems.[1,2] It includes patients with both mild—for example, cutaneous mastocytosis and indolent systemic mastocytosis (ISM)—and more aggressive—for example, aggressive SM (ASM) and MC leukemia (MCL)–forms of the disease, together with cases that have an associated clonal non-MC lineage disease (SM-AHNMD).[1,2] Recently, two additionally rare but clinically and molecularly distinct subvariants of ISM have been identified, which have relevant therapeutic implications: (i) ISM without skin lesions (ISM[s−])[3–5] and (ii) well-differentiated SM.[6–8]

Despite the clinical, biological and prognostic heterogeneity found among the different subtypes of SM, in the great majority (>90%) of cases, the clonal nature of MC is demonstrated by the presence of the stem cell growth factor receptor gene (*KIT*) D816V mutation,[9] except among well-differentiated SM patients; thus, the presence of *KIT* mutation itself, is of relatively limited value for the classification of the disease.[1,9] Interestingly, recent studies[9] showed that poor-prognosis SM, including ASM, MCL and SM-AHNMD[2,10] frequently (≈80% of the cases) carry the D816V *KIT* mutation in myeloid or both myeloid and lymphoid hematopoietic cell compartments other than bone marrow mast cells (BMMC).[9] Noteworthy, BMMC from these later subgroups of SM patients exhibit a unique and more immature immunophenotypic

profile—for example, increased expression of HLA-DR and CD123, and decreased reactivity for CD117, FcεRI and cytoplasmic (Cy) tryptase[11–14]— vs other subtypes of SM in which the *KIT* mutation is typically restricted to BMMC (for example, ISM[s−]) with a mature activated MC phenotype, and increased expression of multiple activation associated-markers (for example, CD63, CD69 and CD203c).[14]

As in ISM[s−], the majority of ISM patients who have skin lesions (ISM[s+]) typically carry the *KIT* mutation restricted to the MC compartment; nevertheless, in around 20% of these cases[9] the *KIT* mutation is also detected in other myeloid or both myeloid and lymphoid cell compartments. Noteworthy, the extent of involvement of hematopoiesis by the *KIT* mutation has been recently reported to be the most powerful independent prognostic criteria for progression of ISM to more aggressive disease—for example, ASM, myelodysplastic syndrome or acute myeloblastic leukemia.[15] However, this information requires complex molecular analyses of highly purified FACS (fluorescence-activated cell sorting)-sorted BM cell populations,[9,15] which limits its routine use out of highly specialized reference centers. Despite all the above, no study has been reported so far, in which the potential association between the immunophenotype of BMMC and multilineage involvement by the *KIT* mutation has been investigated, in order to determine the potential utility of the flow cytometric assessment of the immunophenotypic profile of BMMC in the

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diagnostic screening of multilineage involvement of hematopoiesis by the *KIT* mutation in SM.

In this study, we analyzed the flow cytometric PEP of BMMC from 206 SM patients, and compared it among individuals classified according to the WHO disease subtype, as well as the grade of involvement of BM hematopoiesis by the *KIT* D816V mutation. Our results show that SM patients who carry the *KIT* D816V mutation in BM cell compartments, other than MC, share a unique PEP on their BMMC, independently of the diagnostic subtype of the disease. These results suggest that immunophenotype of BMMC may be used as a surrogate marker for the diagnostic screening of multilineage *KIT* mutation in ISM.

MATERIALS AND METHODS

Patients

Only adult patients (>18 years), who gave their informed consent and fulfilled the WHO, and more recent criteria[1,2] for the diagnosis of ISM[s−], ISM[s+] or ASM (including those cases with AHNMD) were included in the study; additionally, those patients who carried a *KIT* mutation distinct from the D816V or in whom the *KIT* mutation could not be investigated in all the subpopulations of nucleated BM cells, were excluded from further analyses. Overall, 206 BM samples obtained from an identical number of SM patients (141 ISM[s+], 18 ASM and 47 ISM[s−]) who fulfilled the inclusion criteria (111 males and 95 females; median age of 44 years, ranging from 21 to 77 years), and who were diagnosed at the reference centres of the Spanish Network of Mastocytosis (REMA) (Mast Cell Unit of the Hospital Virgen del Valle, Toledo, and Cytometry Service of the Cancer Research Centre, Salamanca, Spain) were studied. In addition, 70 control BM donors (27 males and 43 females; median age of 47 years, ranging from 25 to 77 years), which consisted of 5 healthy subjects and 65 patients undergoing BM aspiration for clinical reasons other than mastocytosis, were also analysed. Of note, a subset of 55 patients and 20 control BM

donors included in this study were also previously described in reference Teodosio *et al*.[14] In all cases, informed consent was obtained prior to the study, according to the guidelines of the local ethics committees and review boards of the two centres.

Multiparameter flow cytometry immunophenotype of BMMC

Multiparameter flow cytometry immunophenotypic studies were performed on BM aspirate samples, processed as previously described,[14,16] using either a FACSCalibur (Becton Dickinson–BD, San Jose, CA, USA) or a FACSCanto II flow cytometer (BDB), equipped with the CellQUEST and FACSDiva software programs (BDB), respectively. Four-color, fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein (PerCP)–cyanin 5.5 (Cy5.5)/allophycocyanin, combinations of monoclonal antibodies (MAb) were used to stain BM cells for a broad set of proteins (Table 1). For those BM samples measured in the FACSCanto II flow cytometer (*n* = 51), a six-color, Pacific Blue/Pacific Orange /FITC/PE/PerCP Cy5.5/PE–cyanin 7 (Cy7), combination of MAb was used (Table 1). In both groups of cases, a double-step acquisition procedure was applied, as recommended by the REMA.[17] For data analysis the INFINICYT software (Cytognos SL, Salamanca, Spain) was used.

Serum tryptase levels

Serum tryptase (sT) levels were determined using the Phadia ImmunoCAP Tryptase System (Phadia, Uppsala, Sweden), according to the instructions of the manufacturer.

Purification of MC and other subpopulations of BM nucleated cells, and detection of *KIT* D816V mutation

Isolation of MC and other subpopulations of nucleated BM cells was performed using a four-way fluorescence-activated cell sorter (FACSAria, BDB), equipped with the FACSDiva software (BDB) as described elsewhere.[9] The *KIT* D816V mutation was detected using a peptide nucleic acid-mediated PCR-clamping technique, on highly-purified fractions (⩾97%

Table 1. List of antibodies used for the immunophenotypic analysis of bone marrow mast cells

Functional group	Ab conjugate	Clone	Commercial source
Activation markers	CD63–FITC	CLBGran/12	Beckman Coulter[a]
	CD69–PE	L78	BD Biosciences[b]
	CD203c–PE	97A6	Beckman Coulter[a]
Adhesion molecules	CD2–FITC	S5.2	BD Biosciences[b]
	CD2–Pacific Blue	TS1/8	Biolegend[d]
	CD22–PE	S-HCL-1	BD Biosciences[b]
Apoptosis-related proteins	Anti-Bcl2–FITC	124	Dako[c]
Complement-associated proteins	CD59–PE	p282 (H19)	BD Biosciences[b]
Cytokine receptors	CD25–PE	2A3	BD Biosciences[b]
	CD117–PE Cy7	104D2D1	Beckman Coulter[a]
	CD117–APC	YB5.B8	BD Biosciences[b]
	CD123–PE	9F5	BD Biosciences[b]
Ig receptors	CD16–PE	3G8	Beckman Coulter[a]
	CD32–PE	AT-10	Cytognos[e]
	CD64–PE	022CL-3	Beckman Coulter[a]
	FcεRI–FITC	Polyclonal	Invitrogen[f]
Major histocompatibility complex	HLA-I–FITC	B9.12.1	Beckman Coulter[a]
(MHC)-associated molecules	HLA-DR–FITC	L234	BD Biosciences[b]
	HLA-DQ–FITC	TÜ169	BD Biosciences[b]
MC enzymes	Total tryptase–FITC	B12	LB Scwhartz[g]
	Mature tryptase–FITC	G5	LB Scwhartz[g]
	Carboxypeptidase A–FITC	CA2	AF Walls[h]
Tetraspanins	CD9–FITC	ALB6	Beckman Coulter[a]
Other proteins	CD34–FITC	8G12	BD Biosciences[b]
	CD34–PerCP Cy5.5	8G12	BD Biosciences[b]
	CD45–Pacific Blue	T29/33	Dako[c]
	CD45–Pacific Orange	HI30	Invitrogen[f]
	CD45–PerCP Cy5.5	2D1	BD Biosciences[b]

Abbreviations: Ab, antibodies; Cy, cytoplasmic; MC, mast cells; PE, phycoerythrin. [a]Beckman Coulter (Beckman Coulter, Miami, FL, USA). [b]BD Biosciences (BDB, San José, CA, USA). [c]Dako (Dako, Glostrup, Denmark). [d]Biolegend (Biolegend, San Diego, CA, USA). [e]Cytognos (Cytognos SL, Salamanca, Spain). [f]Invitrogen (Invitrogen, Carlsbad, CA, USA). [g]This MAb was provided by Dr LB Schwartz (Richmond, VA, USA). [h]This MAb was provided by Dr AF Walls (Southampton, UK).

purity) of BMMC, eosinophils, monocytes, neutrophils, CD34^{+} hemato-poietic precursor cells and lymphocytes, as previously described;[9] in these later fractions no contamination by phenotypically aberrant MC was detected (<0.01%).

Statistical methods, hierarchical clustering analysis and class prediction algorithm

For all continuous variables, their median, mean and s.d., as well as range and both the 25th and 75th, and the 10th and 90th percentiles were calculated; for categorical variables, frequencies were reported. Statistical significance (P value $\leqslant 0.05$) was determined employing the non-parametric Kruskal–Wallis and Mann–Whitney U tests (for continuous variables) or the Pearson's Chi-square test, the binomial test and Fisher's exact test (for categorical variables). Progression-free survival (PFS) curves were plotted according to the method of Kaplan–Meier and survival curves were compared using the log-rank test. For all statistical analyses the SPSS 15.0 software (SPSS, Chicago, IL, USA), was used.

Unsupervised hierarchical clustering analysis was performed using standard correlation coefficients (Pearson correlation) and the average linkage clustering method. Normalization of the datasets was performed by calculating a ratio between the mean fluorescence intensity obtained for each marker and the median mean fluorescence intensity value obtained for that marker in all the samples tested. A logarithmic (base 2) transformation was applied to the ratio values, and the log2 ratios were then used for hierarchical clustering analyses (Cluster 3.0 and Tree View software; Stanford University, Stanford, CA, USA).

In order to explore the predictive value of the different immunopheno-typic features for the detection of cases with multilineage *KIT* mutation, a score-based class prediction model was built, employing a training set of ISM[s+] patients. For the identification of the most discriminant parameters and categorization of continuous variables, receiver operating characteristic (ROC) curves were applied. Those variables that did not reach statistical significance ($P > 0.05$), had little discriminating value (area under the curve (AUC) < 0.6), and/or were tested in $< 80\%$ of the samples analyzed, were excluded from the model. Normalization of each immunophenotypic variable was performed by calculating the ratio between individual mean fluorescence intensity values of SM patients and the median mean fluorescence intensity value obtained for a reference group of normal BMMC, stained and measured under identical conditions as that of the SM patients' samples. The performance of individual markers for the classifica-tion of SM patients with multilineage *KIT* D816V mutation vs patients with *KIT* mutation restricted to BMMC was determined using ROC curves. Accordingly, a score of two was assigned to those variables with higher discriminating power (AUC > 0.7), whereas for the remaining informative variables a score of one was attributed. The result of the sum of the score for the five parameters included in the model was assigned as the score of each individual sample; then the most discriminant score cutoff for *KIT* multilineage involvement was determined using a ROC curve. This classifier was then used to predict the class in a validation set of samples stained with the same combination of MAbs, but measured in a different four-color flow cytometer (validation set no. 1, that corresponds to the subset of 55 patients and 20 control BM donors, which were previously described in Teodosio *et al.*[14]), and a second validation set of patient samples stained with six-color combinations of MAbs and measured in a FACSCanto instrument (validation set no. 2). Sensitivity was calculated as true positive (TP)/(TP + false negative), specificity as true negative (TN)/(TN + false positive), positive predictive value as TP/(TP + false positive), negative predictive value as TN/(TN + false negative), false positive rate as 1−specificity and false negative rate as 1−sensitivity.

RESULTS

Unsupervised hierarchical clustering-based classification of SM patients according to the load and immunophenotypic features of BMMC

In a first step, unsupervised hierarchical clustering analysis was performed on 112 SM patients, who were simultaneously tested for a screening panel of a limited number of parameters ($n = 11$)

(stained and measured under the same conditions). On the basis of this analysis, patients were classified into two major groups (groups A and B in Figure 1a): one group (group A) included most ASM cases (11/12 (92%; $P = 0.006$)), whereas the other (group B) comprised the majority of ISM[s−] patients (25/27 (93%; $P < 0.0001$)). Interestingly, ISM[s+] patients were equally distributed in both groups: 49% in group A and 51% in group B ($P = 0.91$). Nevertheless, when the pattern of BM involvement by the *KIT* mutation was considered, group A included the majority of SM patients with myeloid, and myeloid plus lymphoid *KIT* mutation (14/19 (74%; $P = 0.04$) and 12/15 (80%; $P = 0.02$), respectively). Conversely, group B included 55/78 (71%; $P < 0.0001$) cases with the *KIT* mutation restricted to BMMC (Figure 1a). In order to further confirm these results, a similar analysis with a larger number of phenotypic parameters was performed, for a subset of 34 patients (Figure 1b) and a separate new set of patients ($n = 35$), evaluated in a distinct FACSCalibur flow cytometer instrument (Figure 1c). Both groups displayed clustering profiles, which were similar to those observed for the first group of patients (Figure 1a).

Flow cytometric protein expression profiles of BMMC from ISM[s+] patients with different degree of BM involvement by the D816V *KIT* mutation

In detail, ISM patients with involvement of multiple myeloid and/or lymphoid BM cell compartments by the *KIT* mutation displayed increased numbers of phenotypically aberrant CD25^{+} BMMC ($P = 0.008$), and higher sT levels ($P = 0.01$) (Figure 2), with values closer to those detected in ASM vs both ISM[s−] and ISM[s+] cases with *KIT* mutation restricted to BMMC. Furthermore, coexistence of normal and aberrant BMMC populations was restricted to SM patients with *KIT* mutation restricted to the BMMC compartment (17.6% vs 0%; $P = 0.0004$). In addition, an increasing number of mutated BM cell compartments were associated with significantly ($P < 0.05$) lower light scatter values, reflected also by a decreased content of cytoplasmic MC enzymes, such as carboxypeptidase A and total tryptase (Figure 2). In line with this, expression of other maturation-associated markers, such as CD203c, the high-affinity IgE receptor (FcεRI) ($P = 0.02$), CD45 ($P = 0.01$) and CD9 ($P = 0.009$), was also significantly lower among cases with mutilineage *KIT* mutation. Interestingly, ISM[s+] cases with both myeloid and lymphoid BM involvement displayed slightly (but not significantly) decreased expression of the CD63 and CD69 activation markers (Figure 2).

Performance of the prediction algorithm for the classification of SM patients

On the basis of the above findings, an algorithm, for the classification of SM patients with multilineage *KIT* D816V mutation vs patients with *KIT* mutation restricted to BMMC, was built (Figure 3). For the development of the algorithm, an immuno-phenotypic score was established using those phenotypic markers (% of BMMC, FSC, SSC, CD45 and FcεRI), which were tested in $> 80\%$ of the patients, and had a high individual discrimination power (AUC > 0.6; $P < 0.05$) between BMMC from ISM[s+] cases with *KIT* mutation restricted to MC and cases with multilineage involvement, and a training set of ISM[s+] patients in which all these variables were simultaneously analysed ($n = 75$) (Table 2). Using this algorithm, patients with multilineage *KIT* mutation were identified based on aberrant expression of CD25 by BMMC, absence of a coexisting normal BMMC population and an immunophenotypic score > 3 (Figure 3). This approach displayed a high efficiency to classify patients according to the degree of involvement of hematopoiesis by the *KIT* mutation, with high sensitivity (range: 80–92%) and specificity (range: 71–94%) when assessed in both the training and the two validation groups of SM patients; similar results were found when ISM[s+] patients, and the overall group of SM patients were considered, which proves the
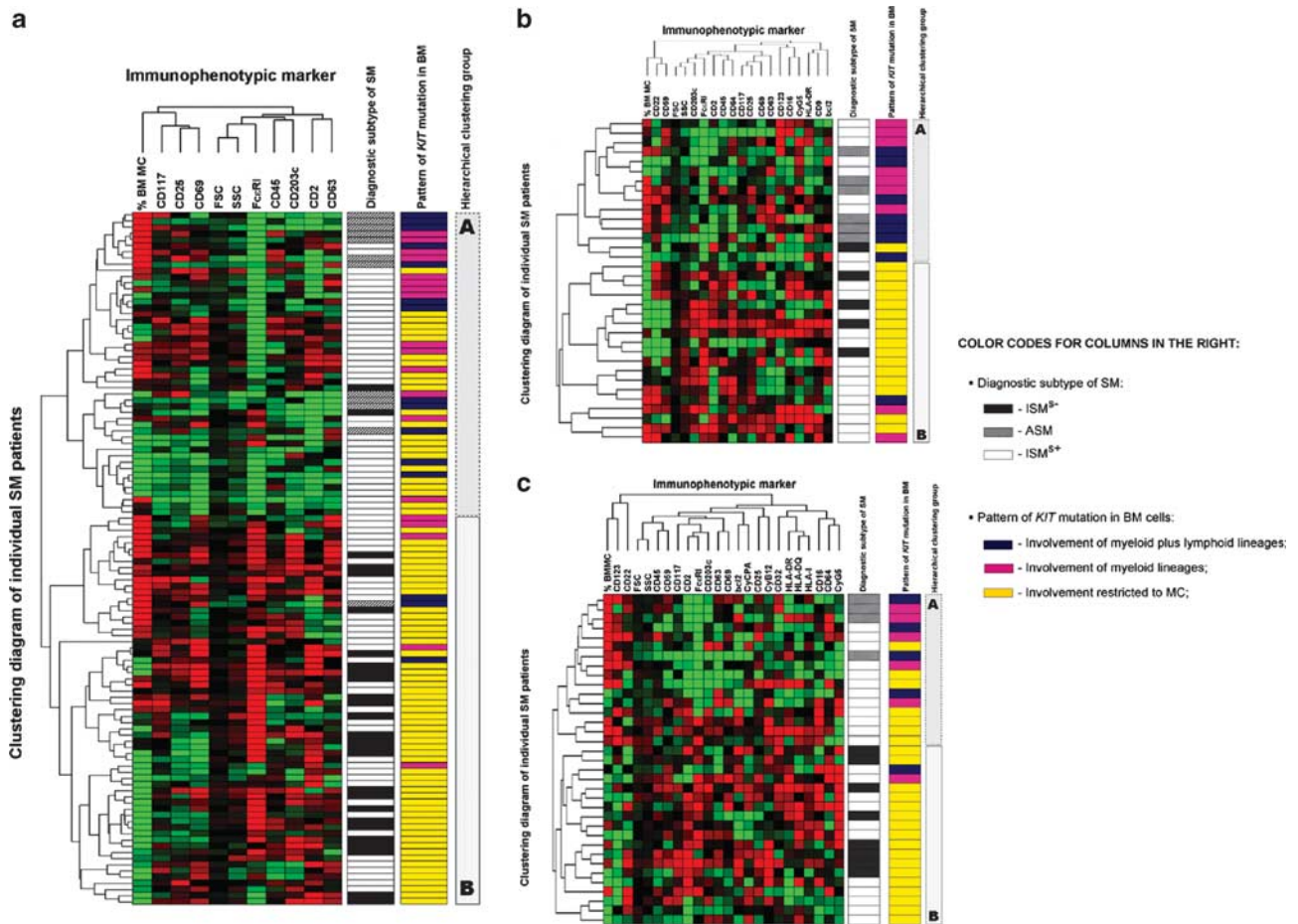
Figure 1. Hierarchical clustering analysis of systemic mastocytosis (SM) bone marrow (BM) samples based on the protein expression profiles (PEP) of bone marrow mast cells (BMMC), and the BMMC load in three distinct sets of SM patients diagnosed with different subtypes of SM and distinct degree of involvement of different compartments of BM cells by the *KIT* D816V mutation. In panels (**a**) and (**b**) a series of 112 patients tested for a small screening panel (11 parameters) and a subset of 34 patients, in which a broader set of proteins was studied (20 parameters) are displayed, respectively. Panel (**c**) shows a different set of patients (validation set; $n = 35$) analyzed for 24 different parameters, including most of the markers tested in the previous two groups of patients. Rows represent individual BM samples or patients, whereas columns represent the normalized log2 ratios of the percentage of BMMC, and the mean fluorescence intensity obtained for each marker divided by the median value obtained for that parameter in all samples tested.

efficiency of the proposed algorithm in SM, independently of its specific diagnostic subtype (Table 3).

Prognostic impact of the PEP of BMMC on PFS of ISM^{s+} patients
Once the potential impact of PEP of BMMC from ISM^{s+} patients on patient outcome was investigated in a subset of 116 ISM^{s+} patients with a follow-up of $\geqslant 22$ months (median of 126 months (range: 22–596 months) after disease onset), we found a significant adverse impact on PFS for an immature BMMC phenotype, as defined by the proposed algorithm (based on the aberrant expression of CD25 and BMMC phenotypic score > 3, in absence of a coexisting normal BMMC population) (Figure 4a). Five patients (4%) who showed signs of progression according to Escribano *et al.*[15] 60, 76, 249, 310 and 428 months after disease onset, also had myeloid ($n = 2$) or myeloid plus lymphoid ($n = 3$) BM involvement by the *KIT* D816V mutation; thus, the phenotypic score showed a very similar impact on PFS to that of the presence of multilineage *KIT* D816V mutation ($P = 0.001$ in both cases).

DISCUSSION
SM includes a group of clinical, biological and prognostically heterogeneous disorders,[2] which typically carry in common the

D816V activating *KIT* mutation in phenotypically aberrant BMMC.[9] Despite the fact that the presence of *KIT* mutation has limited value for the classification of SM, it has been shown that BMMC from aggressive forms of mastocytosis (for example, ASM and MCL), systematically carry this mutation in myeloid or both myeloid and lymphoid hematopoietic cell compartments other than BMMC,[9] at the same time they display PEP associated with an increased MC immaturity.[14] Furthermore, multilineage BM involvement by the D816V *KIT* mutation has recently been shown to identify a subset of ISM^{s+} patients with increased risk of progression to more aggressive disease subtypes (for example, ASM, myelodysplastic syndrome or acute myeloblastic leukemia).[15] Despite these findings, no study has been reported so far, in which the potential association between the immunophenotype of BMMC and multi-lineage *KIT* mutation involvement has been investigated, particularly among ISM patients. This is of special relevance because current approaches to determine multilineage involvement of hematopoietic cells in SM by the *KIT* mutation require rather complex and time consuming technical procedures, which are not widely available for routine use (for example, flow cytometry sorting of multiple cell populations for mutational analysis).[9,15] Here, we analyzed the PEP of BMMC from a large series of SM patients with a broad panel of proteins, in order to determine its association with the degree of involvement of different hematopoietic BM cell
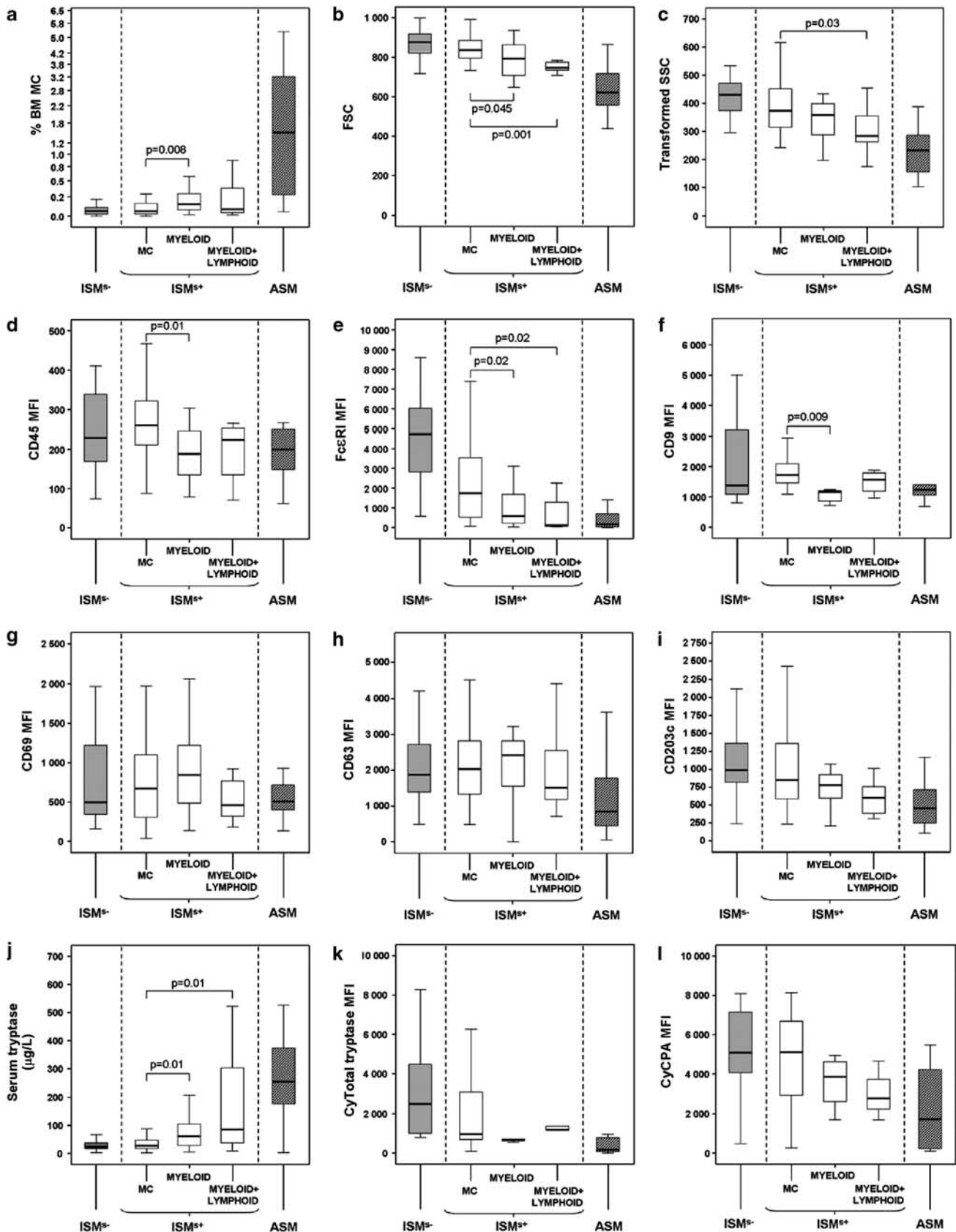
Figure 2. Bone marrow mast cells (BMMC) load (percentage of cells) (**a**), scatter (**b**, **c**) and immunophenotypic characteristics of BMMC (**d**–**i**), serum (μg/l), cytoplasmic total tryptase levels and the expression pattern for cytoplasmic carboxypeptidase A (**j**–**l**) in systemic mastocytosis (SM) patients ($n = 194$), classified according to the diagnostic subtype of the disease (ISM^{s-}, ISM^{s+} and aggressive SM (ASM)), and the degree of involvement of BM hematopoietic cell lineages by the *KIT* D816V mutation (restricted to BMMC or extended to other myeloid or myeloid and lymphoid BM cells). ISM, indolent SM; MFI, mean fluorescence intensity per BMMC.

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compartments by the *KIT* D816V mutation, and its potential utility as a surrogate marker for multilineage BM involvement.

Overall, our results show that SM patients who carry the *KIT* D816V mutation in BM cell compartments other than MC, share unique immunophenotypic features, regardless of the diagnostic subtype of the disease. In line with this, BMMC from ISM[s+] patients with *KIT* mutation restricted to MC exhibit an immuno-phenotypic profile similar to that of other good-prognosis subtypes of SM (with the *KIT* mutation also restricted to MC), like ISM[s−]; similar to the latter cases, this group of ISM[s+] patients also displayed a mature (FcεRI[hi]), activated (for example, high expression of the CD203c activation marker) BMMC immuno-phenotype.[14,18,19] Conversely, ISM[s+] patients with multilineage involvement shared immunophenotypic features with ASM,[14] which typically also carry myeloid or myeloid plus lymphoid BM involvement by the *KIT* mutation.[9] In these ISM[s+] cases, like in ASM patients, an increasing number of BM cell populations bearing the mutation was also associated with a higher BMMC load and sT levels. Likewise, BMMC from ISM[s+] cases with multilineage involvement also displayed decreased expression of proteins known to be acquired during the BMMC maturation process, like FcεRI, CD45 and cytoplasmic MC enzymes (for example, carboxypeptidase A and total tryptase), associated with decreased light scatter features (FSC, SSC), further confirming the immature PEP of their BMMC.[13,14] Overall, these results suggest that in SM, the occurrence of an extended clonal hematopoiesis with multilineage involvement by *KIT* mutated cells could be associated with an early blockade of MC maturation, leading to more immature BMMC features and a less activated phenotype, in association with an increased MC load. As all ISM[s+] cases carry in common an activating *KIT* mutation,[20] in principle, the mutation itself cannot fully explain such phenotypic differences; further studies are necessary to elucidate whether such maturation blockade is the result of the coexistence of additional genetic and/or epigenetic events in the D816V[+] hematopoietic precursor cell, as previously suggested for polycythemia vera, essential thrombocythemia and primary myelofibrosis cases, which share an identical activating somatic mutation of the *JAK2* tyrosine kinase gene (*JAK2*[V617F]), but display distinct clinico-biological features.[21,22] Alternatively, the *KIT* mutation could target a different stem or precursor cell in both groups of ISM[s+] patients with an increased predisposition of ISM[s+] cases with multilineage involvement, to acquire additional genetic events and progression to more aggressive forms of the disease.[15]

This association, between the BMMC immunophenotype and the degree of BM involvement by the D816V *KIT* mutation, was further confirmed by the prediction algorithm developed on the basis of those variables that were identified among ISM[s+] patients to discriminate between cases with MC-only vs extensive BM involvement by the *KIT* mutation: aberrant expression of CD25 in association with a FcεRI^{lo}, FSC^{lo}, SSC^{lo} and CD45^{lo} immature phenotype of BMMC, in the absence of coexisting BMMC with a normal phenotype. The proposed algorithm showed a high efficiency in classifying not only ISM[s+] patients but also other SM patients, into cases with MC-restricted vs multilineage
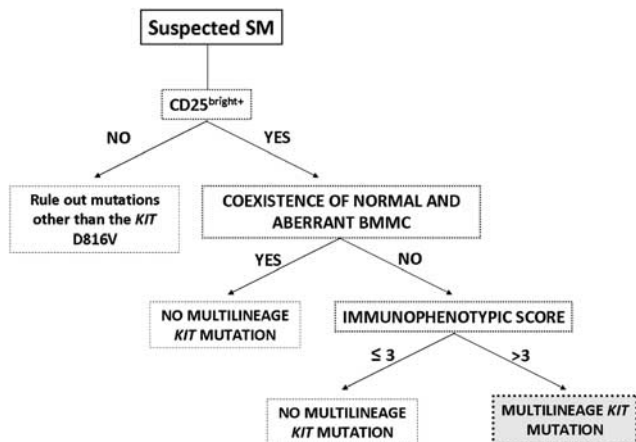

Figure 3. Algorithm developed and proposed for the classification of systemic mastocytosis (SM) patients with multilineage *KIT* D816V mutation vs patients with *KIT* mutation restricted to bone marrow mast cells (BMMC).

Table 2. Performance of individual markers and cutoff values for the classification of systemic mastocytosis patients with multilineage *KIT* D816V mutation vs patients with *KIT* mutation restricted to the bone marrow mast cell compartment, as assessed by receiver operating characteristic (ROC) curves

Parameter	AUC	P-value	Cutoff	Sensitivity (%)	Specificity (%)
BMMC load (%)	0.648	0.010	⩾0.1150	59	68
FSC (ratio)	0.713	0.001	⩽1.2956	57	85
SSC (ratio)	0.660	0.016	⩽1.4851	71	56
CD45 (ratio)	0.684	0.006	⩽1.9921	86	48
FcεRI (ratio)	0.712	0.004	⩽1.1417	48	89

Abbreviations: AUC, area under the curve, BMMC, bone marrow mast cells.

Table 3. Performance of the prediction algorithm developed for the identification of systemic mastocytosis patients with multilineage *KIT* D816V mutation vs patients with *KIT* mutation restricted to the bone marrow mast cell compartment

Parameters	Training set (%)		Validation set 1 (%)		Validation set 2 (%)	
	ISM[s+] (n = 75)	Overall SM patients (n = 116)	ISM[s+] (n = 41)	Overall SM patients (n = 55)	ISM[s+] (n = 15)	Overall SM patients (n = 20)
Sensitivity	83	86	80	89	88	92
Specificity	79	83	93	94	71	75
Positive predictive value (PPV)	61	69	57	73	78	85
Negative predictive value (NPV)	92	93	97	98	83	86
False-positive rate (FPR)	21	18	8	7	29	25
False-negative rate (FNR)	17	14	20	11	13	8

Abbreviations: ISM[s+], indolent systemic mastocytosis with skin lesion; MC, mast cell; SM, systemic mastocytosis.
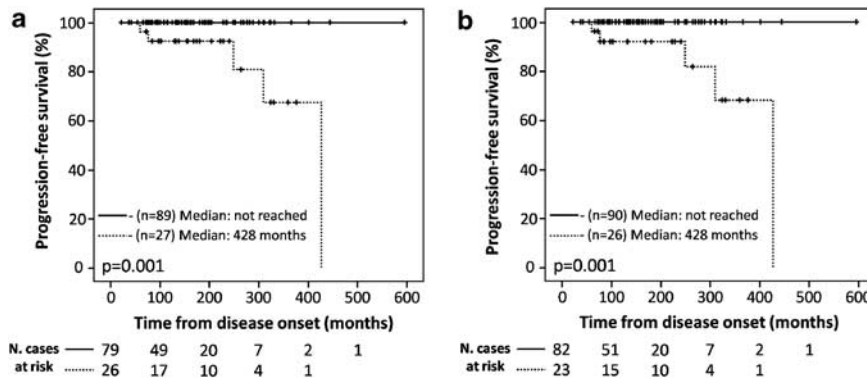
Figure 4. Progression-free survival curves of ISM^{s+} patients ($n = 116$), classified according to the presence (dotted line) or absence (full line) of multilineage involvement of hematopoiesis by the *KIT* D816V mutation, as determined by applying the prediction algorithm based on the immunophenotypic features of bone marrow mast cells (**a**) or using molecular biology techniques (**b**). ISM, indolent systemic mastocytosis.

involvement of BM hematopoietic cells by the *KIT* mutation, regardless of the diagnostic subtype of the disease. Such approach can be easily implemented in routine diagnostic flow cytometry laboratories and does not require generation of a previous reference group of BM samples from SM patients, as it is based on the phenotypic features of normal or reactive BM samples. Nevertheless, it shoud be noted that differences in the sensitivity and specificity of the algorithm were observed between validation groups I and II; such differences may be due to the different number of patients included in each group ($n = 55$ vs 20, respectively), and/or the distribution of the patients within the two groups, as the group I included only 16% ($n = 9/55$) patients with mutlilineage *KIT* mutation, whereas these cases represented 60% ($n = 12/20$) of the patients in group II; however, only few patients were misclassified in both validation sets I and II ($n = 4$ and 3 cases, respectively). Furthermore, detection of mutilineage *KIT* D816V mutation using the proposed surrogate PEP algorithm had a significant impact on PFS of ISM^{s+} patients, identical to that provided by the investigation of the presence of the *KIT* mutation on purified cell populations; this is because none of the discrepant cases progressed at the moment of closing this study. Altogether, these findings support the utility of immunophenotyping of BMMC as a surrogate marker to predict the degree of clonal hematopoiesis in the BM of SM patients, independently of the clinical subtype of the disease.

In summary, our results show that the immunophenotypic profile of BMMC from ISM patients is significantly associated with the degree of BM involvement by the D816V *KIT* mutation, as more immature BMMC features are typically found in ISM^{s+} cases who carry multilineage clonal hematopoiesis; these findings suggest that the immunophenotype of BMMC may be used as a surrogate marker for the diagnostic screening of multilineage *KIT* mutation among ISM^{s+}, and therefore, prognostic stratification of this group of ISM patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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3.3. **THE GENE EXPRESSION PROFILE OF HIGHLY-PURIFIED BONE MARROW MAST**

CELLS IN SYSTEMIC MASTOCYTOSIS

Journal of Allergy and Clinical Immunology (under revision)

Teodosio C. performed the RNA extraction and MFC validation experiments, analyzed/interpreted the data, made the figures and wrote the paper.

Gene expression profile of highly-purified bone marrow mast cells in systemic mastocytosis

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ABSTRACT

Background: Despite the great majority (>90%) of systemic mastocytosis (SM) patients carry a common genetic lesion - the *KIT* D816V mutation -, little is known regarding the molecular and biological pathways underlying the clinical heterogeneity of the disease.

Objective: To analyze the gene expression profiles (GEP) of (highly-purified) bone marrow (BM) mast cells (MC) in SM and its association with the distinct clinical variants of the disease.

Methods: Gene expression analyses were performed in highly-purified BMMC from SM patients carrying the D816V *KIT* mutation (n=26) classified according to the diagnostic subtype of SM vs. normal/reactive BMMC (n=5) by DNA-oligonucleotide microarrays. Validation of GEP results was performed using flow cytometry in the same set of samples and in an independent cohort of 134 individuals.

Results: Overall, 915 transcripts were significantly deregulated in SM patients, with a common GEP (n=286 genes) for all subvariants of SM analyzed, characterized by up-regulation of genes involved in the innate and inflammatory immune response, including interferon-induced genes and genes involved in cellular responses to viral antigens, together with complement inhibitory molecules. Most interestingly, distinct GEP were observed among the different subtypes of SM, reflecting an increased lipid metabolism vs. increased transcription and protein processing in indolent SM (ISM) without skin lesions vs. ISM with skin lesion, and deregulation of apoptosis and cell cycle in aggressive SM.

Conclusion: BMMC from patients with different clinical subtypes of SM display distinct GEP, which may reflect new targeted pathways involved in the pathogenesis of the disease.

Key messages:

D816V *KIT* mutation in SM bone marrow MCs is associated with up-regulation of genes involved in innate and inflammatory immune responses.

Different subtypes of SM exhibit distinct gene expression profiles, which reflect an increased lipid vs. protein metabolism in indolent SM without skin lesions vs. indolent SM with skin lesions, respectively, and deregulation of apoptosis and cell cycle in aggressive SM.

Capsule summary:

Gene expression microarray analyses performed on highly-purified bone marrow mast cells show distinct gene expression profiles in systemic mastocytosis patients with distinct clinical variants of the disease, carrying the same D816V *KIT* mutation.

Key words:

Systemic mastocytosis, D816V *KIT* mutation, gene expression profile, mast cells, cDNA microarrays.

Abbreviations:

SM: Systemic mastocytosis;

MC: Mast cell;

BM: Bone marrow;

BMMC: Bone marrow mast cell;

ISMs-: Indolent systemic mastocytosis without skin lesion;

ISMs+: Indolent systemic mastocytosis with skin lesion;

ASM: Aggressive systemic mastocytosis;

GEP: Gene expression profile;

FDR: False discovery rate;

INTRODUCTION

Systemic mastocytosis (SM) comprises an heterogeneous group of disorders characterized by abnormal growth and accumulation of clonal mast cells (MC) in one or more organ systems.[1, 2] Based on their clinicopathological features, seven variants of SM are currently recognized by the World Health Organization (WHO), including mild (e.g. indolent SM - ISM) and aggressive (e.g. aggressive SM –ASM – and mast cell leukemia - MCL) forms of the disease.[1, 2] In addition, recent studies point out the existence of two additional subvariants of ISM - well-differentiated SM (WDSM) - and ISM without skin lesion (ISMs-)-, with unique clinico-biological and immunophenotypical features, and different therapeutic requirements.[3-8] Despite these distinct subtypes of SM are clinically well-defined entities, little is known about the molecular mechanisms, biological functions and pathways underlying disease heterogeneity, as the great majority (>90%) of these patients (except WDSM) carry a common genetic lesion, the *KIT* D816V activating mutation.[9]

Gene expression profiling using cDNA microarray technology has largely contributed to a better understanding of the molecular mechanisms underlying normal and altered biological processes in many disease conditions.[10, 11] However, reports about the cDNA microarray analysis of gene expression profiles (GEP) of SM are scarce and they have either considered SM as a single entity[12] or focused on single disease subvariants – e.g. ISM -.[13, 14] Most importantly, in all such studies, either BM mononuclear cells[12] or whole peripheral blood samples,[13, 14] containing variable (typically low) percentages of MC were used to assess GEP; indeed, none of them specifically investigated the GEP of highly-purified BMMC fractions, therefore providing biased and complex results which are difficult to interpret depending on the degree of BM infiltration by MC and the variable distribution within individual samples of other different BM and PB cell populations.[15, 16]

In the present study we analyzed for the first time the GEP of highly-purified BMMC from a relatively large cohort of patients with distinct subtypes of indolent and aggressive SM who carried in common the D816V *KIT* mutation, *versus* normal/reactive BMMC. Overall, our results show a common GEP for all subvariants of SM analyzed, and unique GEP specific for each of the subtypes of SM analyzed.

MATERIAL AND METHODS

Patients. BM aspirates were obtained from 102 patients (56 males and 46 females; median age of 52 years, ranging from 24 to 82 years) diagnosed with SM at the reference centres of the Spanish Network on Mastocytosis (REMA) (Mast Cell Unit of the Hospital Virgen del Valle, Toledo and; Cytometry Service of the Cancer Research Centre, Salamanca, Spain).[1, 2] Patients were classified according to the WHO criteria[1, 2] and more recent recommendations,[4-6] as ISM without skin lesion (ISMs-), 22 cases; ISM with skin lesion (ISMs+), 62, and; ASM, 18. Additionally, control BM aspirates were obtained from 63 individuals (32 males and 31 females; median age of 50 years, ranging from 23 to 89 years), including 8 healthy donors (normal BM) and 55 individuals undergoing BM aspiration for clinical reasons other than mastocytosis or myeloid neoplasm. In all cases, informed consent was obtained, according to the guidelines of the local Ethical Committees of the two centres.

Isolation of MC and other subpopulations of BM nucleated cells, DNA extraction and detection of the D816V _KIT_ mutation. Isolation of MC and other subpopulations of BM cells, was performed as previously described in detail[9, 17] (see Methods section in the Online Repository).

RNA extraction, microarray hybridization and data analysis. Assessment of GEP was performed on RNA extracted from purified BMMC from 31 individuals, including 5 controls (3 healthy donors and 2 individuals carrying a B-cell lymphoproliferative disorder which exhibited reactive mast cell hyperplasia – cases #4 and #5) and 26 SM patients (Table I), using the Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) (see Methods section in the Online Repository).

For data analysis, GEP raw data (Affymetrix.CEL files) were normalized by the robust multi-array average (RMA) algorithm, which included background correction, quantile normalization, log2-transformation, and probe set summarization.[18] In order to decrease the risk for false positive results, genes which did not show expression levels ≥20 (arbitrary unlogged units) in at least one sample were removed from further analyses. Two additional filters were

used to select genes for further analyses, for exclusion of those genes which could be altered because of changes in the BM microenvironment (e.g. altered expression in reactive vs. normal BMMC): i) genes which displayed a heterogeneous expression pattern in the control group, defined by a coefficient of variation for unlogged values of >40%, and; ii) genes that showed higher differences between median values in reactive *versus* normal MC, than in reactive *versus* SM MC. Differentially expressed genes between normal/reactive samples and each diagnostic subtype of SM were identified using supervised two-class unpaired Significance Analysis of Microarray (SAM),[19] assessed by the MultiExperiment Viewer (MeV) v.4.6.1 software,[20] with a combined cutoff for false discovery rate (FDR <0.01) and absolute fold change (≥2.0).

For gene ontology (GO) term annotation and functional enrichment analysis of significantly deregulated genes, the GeneCodis (Gene Annotation Co-occurrence Discovery)[21] functional annotation tool was used. Analysis of gene interactions and correlation networks was performed using the Ingenuity Pathway Analysis software (Ingenuity Systems®, www.ingenuity.com). For the identification of those genes with predictive value to discriminate between ISM and ASM patients, the PAM (Prediction Analysis for Microarrays) software (version 2.1; University of Stanford, Stanford, CA), based on the nearest shrunken centroids method was applied, using a 10-fold cross-validation approach.[22]

RESULTS

Overall transcription profile of BMMC in SM. Supervised analysis of the GEP of BMMC from SM patients revealed a distinct pattern for each of the three diagnostic subtypes of SM included in the study, but with a common imprint. Overall, ISM cases (ISMs- and ISMs+) showed similar number of deregulated genes - 474 genes (441 up-regulated and 33 down-regulated) in ISMs- and 438 genes (411 up-regulated and 27 down-regulated) among ISMs+ -, whereas ASM patients displayed a more altered GEP with 758 deregulated genes (642 up-regulated and 116 down-regulated), adding up to a total of 915 different deregulated transcripts in SM versus normal/reactive BMMC. Almost one third of the deregulated genes (n=286 genes) were altered in common in the three diagnostic subgroups of SM (ISMs-, ISMs+ and ASM) (Figure 1). Noteworthy, the *IL2RA* gene (IL2 receptor-alpha chain - CD25) was included in this common GEP, as it was significantly up-regulated in all three patient groups (fold-change systematically >10.0), in contrast to the *CD2* adhesion-related molecule, which was only significantly up-regulated in ISMs- patients.

Functional characterization of the deregulated GEP common to ISMs-, ISMs+ and ASM BMMC. Analysis of the biological and functional significance of the deregulated GEP common to BMMC carrying the *KIT* D816V mutation, based on those 286 genes which were significantly altered in all three variants of the disease analyzed, revealed a significant enrichment for genes involved in biological functions mostly related to the immune response (Figure 2). This included genes involved in innate and inflammatory immune responses like *IL1R1* (interleukin 1 receptor, type I), *LIF* (leukemia inhibitory factor), the *CST7* protease regulator (cystatin F), the *CCL23* and *CCL3* chemokines, the *CD4* glycoprotein and complement-associated (regulatory) molecules like *CLU* (clusterin), *CFH* (complement factor H) and *SERPING1* (plasma protease C1 inhibitor) (Figure 2; Table II). Additionally, genes related to signal transduction associated with cytokine-mediated signalling (Table III), such as downstream targets of the Jak/STAT pathway – e.g. *SOCS2* (suppressor of cytokine signaling 2), *BCL2L1* (BCL2-like 1) or the *PIM1* oncogene -, were also up-regulated in common in ISMs-, ISMs+ and ASM (Table II). Furthermore, genes associated with type-I interferon (IFN) signalling (including both IFNγ and IFNα-mediated

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signalling pathways), together with genes involved in anti-viral responses (Table III; Figure 2) were also altered in BMMC from all three groups of SM, as reflected by up-regulation of the *MX1* and *MX2* (interferon-induced GTP-binding proteins Mx1 and Mx2), *OAS1, OAS2* and *OAS3* genes (2'-5'-oligoadenylate synthetases 1, 2 and 3), *IFIT1* and *IFIT3* (interferon-induced protein with tetratricopeptide repeats 1 and 3) genes, as well as the *PML* (promyelocytic leukemia protein) and *IFI44L* (interferon-induced protein 44-like) genes, and cytosolic DNA-sensing pathway genes (Table II) like the *DDX58* (DEAD (Asp-Glu-Ala-Asp) box polypeptide 58) enzyme, respectively.

Functional profile of genes differentially expressed in ISMs-, ISMs+ and ASM BMMC. Functional enrichment analysis of the distinct GEP associated with individual subtypes of SM revealed a significant enrichment in ISM (ISMs- and ISMs+) patients for genes involved in protein phosphorylation (e.g. the c-src tyrosine kinase, *CSK*) and T cell costimulation, including the *TNFRSF14* (tumor necrosis factor receptor superfamily member 14), *PDCD1LG2* (programmed cell death 1 ligand 2) and *CD4* genes and, specifically among ISMs- BMMC, the *HLA-DRB1* and *HLA-DRB5* HLA class II molecules (Table IV).

In turn, ISMs- was specifically characterized by up-regulation of genes related to lipid metabolism, including molecules involved in lipid transport like *APOL4* (Apolipoprotein L, 4), metabolic enzymes like *ACER2* (alkaline ceramidase 2), *PNPLA6* (neuropathy target esterase), *LIPA* (lysosomal acid lipase/cholesteryl ester hydrolase) and *LPIN1* (phosphatidate phosphatase LPIN1), and molecules involved in eiconsanoid and/or prostanglandin metabolism like *FADS1* (fatty acid desaturase 1) and *ACOX1* (peroxisomal acyl-coenzyme A oxidase 1 (Table III). Conversely, ISMs+ BMMC displayed marked up-regulation of the transcriptional machinery (including initiation, elongation and termination) and mRNA capping related genes (e.g. *POLR2K, GTF3C3* or *GTF2E1*) in association with transcripts involved in protein-trafficking - e.g. the *NAPA* (N-ethylmaleimide-sensitive factor attachment protein, alpha) and *GOPC* (golgi-associated PDZ and coiled-coil motif containing) genes-, and post-translational protein modification processes, including protein folding, like the *PPIB* (cyclophilin B), *AHSA1* (activator of 90 kDa heat shock protein ATPase homolog 1) and *MESDC2* (mesoderm development candidate 2) transcripts (Table IV).

Interestingly, both ISMs+ and ASM patients exhibited deregulated expression of adhesion-related molecules, but with distinct profiles: while 19% (5/26) of this group of genes were down-regulated in ASM, only 5% (1/19) showed this pattern of expression in ISMs+ (Tables IV and V). Similarly, a significant enrichment for genes related with apoptosis was observed both among ISMs+ and ASM BMMC - e.g. increased expression of *CASP1*, *CASP10* (caspases 1 and 10), *TNFRSF10B* (TRAIL-R2), *XAF1* (XIAP associated factor 1), *HTATIP2* (HIV-1 Tat interactive protein 2) and *BCL2L1* (BCL2-like 1)-. However, among ASM (but no ISMs+) this was associated with an anti-apoptotic GEP - e.g. up-regulation of the bifunctional apoptosis regulator (*BFAR*) gene and of interleukin 1 beta (*IL1B*) - (Tables IV and V). BMMC from ASM also displayed significant deregulation of multiple cell-cycle related genes, including transcripts involved in the G_1/S and M/G_1 transitions, S-phase and cell-cycle checkpoints, together with genes involved in DNA repair; this included, up-regulation of the *CCNA1* (cyclin A1), *CCNB1* (cyclin B1), *CDC6* (cell division cycle 6 homolog), *CKS2* (CDC28 protein kinase regulatory subunit 2) and *CDK4* (cyclin-dependent kinase 4) cell cycle-associated genes and the *RFC3* - replication factor C (activator 1) 3 - and *POLD3* - polymerase (DNA-directed), delta 3, accessory subunit – DNA repair genes, among other genes (Table V).

Classification of ISM versus ASM patients based on GEP. Supervised class prediction analysis was used to identify those genes that better discriminated between ISM (ISMs- plus ISMs+) and ASM, based on those 915 transcripts significantly deregulated in SM. Overall, 22 genes allowed classification of ISM vs. ASM patients with an overall accuracy of 92% (24/26) (92% and 93% accuracy for ISM and ASM cases, respectively), with only one ISMs+ patient with myeloid plus lymphoid multilineal *KIT* D816V mutation (case #17, Table I) and one ASM patient (case #29, Table I) being misclassified. The majority (68%; 15/22) of these 22 informative genes were up-regulated only in ASM (e.g. the *IL13* and *IL1B* cytokines, the *CD52* glycoprotein and the *CKS2* and *GADD45A* cell cycle related genes), whereas only two genes (9%; 2/22) were specifically altered in ISM patients, - the *COL12A1* (collagen, type XII, alpha 1) adhesion-related molecule and *MYO16* (myosin XVI) - (Figure 3).

DISCUSSION

SM is a clinical, biological and prognostically heterogeneous group of disorders, characterized by an abnormal expansion and accumulation of clonal MC in the BM.[2] Since the majority (>90%) of SM patients carry the same genetic lesion (D816V *KIT* mutation),[9] the presence of the *KIT* mutation itself does not explain the heterogeneous clinical behaviour of the disease. Currently, the molecular mechanisms and pathways underlying the different subtypes of SM remain largely elusive. In order to address this issue, here we analyzed for the first time, the GEP of highly-purified MC isolated from BM samples of SM patients with distinct variants of the disease who carried the D816V *KIT* mutation. Overall, our results show that the distinct subtypes of SM share a common GEP, distinct from that of normal/reactive BMMC; in turn, distinct expression and functional profiles were also detected in ISMs-, ISMs+ and ASM.

Stem cell factor (SCF) signalling through its receptor Kit, plays a key role in the biology of MC, leading to the engagement of multiple downstream signalling pathways involved in MC growth, differentiation, survival, chemotaxis and cytokine production.[23, 24] Interestingly, the activating *KIT* D816V mutation common to all SM patients studied here, translated in a common GEP characterized by generalized up-regulation of genes involved in cytokine-mediated signal transduction, including target genes of the Jak-STAT pathway, like the anti-apoptotic *BCL2L1* gene coding for the Bcl-xL protein, previously described to be strongly expressed by BMMC from SM patients.[25, 26] Within this common GEP, a significant enrichment was also found for type I IFN-dependent response genes, in the absence of interferon therapy. Since most of these IFN-induced genes (e.g. *OAS1, OAS2, MX1, MX2* or *CCL3*) have been previously found to be also up-regulated upon MC infection by viruses,[27, 28] our results could suggest that Kit downstream signalling could play an important role in MC responses to viral infection. In line with this hypothesis, previous studies have reported that infection of endothelial cells by the Kaposi's sarcoma herpesvirus (KSHV) induces up-regulation of Kit, and that the inhibition of either Kit activation or its downstream effectors reverses the KSHV-induced morphological transformation which is characteristic of the infected cells.[29, 30]

Furthermore, an overall enrichment in genes involved in the innate and inflammatory immune response was also detected in ISMs-, ISMs+ and ASM. These included genes coding

for complement regulatory proteins like *CFH* (complement factor H), *SERPING1* (plasma protease C1-inhibitor) and *CLU* (clusterin), the later being previously reported to be up-regulated also in SM BM mononuclear cells.[12] These findings, together with the increased expression of membrane-bound complement inhibitory proteins such as CD35 (complement receptor 1, CR1) and CD59 (membrane inhibitor of reactive lysis, MIRL),[8, 31] could reflect the involvement of Kit signalling in an enhancement of self-defence mechanisms against complement-dependent cytotoxicity in SM, similar to what has been described for other types of tumor cells.[32]

Despite such a GEP was found in common to ISMs-, ISMs+ and ASM, distinct patterns of deregulation of different other biological processes were also found within each of the three diagnostic subtypes of SM. Overall, ISMs- cases displayed a higher number of specifically deregulated genes than ISMs+, including the *CD2* adhesion-molecule, whose protein expression progressively decreased from ISMs- to ISMs+ and ASM, in agreement with a more limited diagnostic sensitivity for CD2 than CD25 particularly among ASM.[8, 33] Functional enrichment analysis of the GEP of ISMs- BMMC revealed specific up-regulation of transcripts involved in lipid metabolism, probably translating in an increased phospholipid metabolism and production of arachidonic acid metabolites (e.g. prostaglandin D2 and leukotriene C4),[34, 35] which would be partially responsible for the MC-mediator related symptoms typically displayed by virtually all ISMs- patients.[4, 6] Conversely, ISMs+ patients specifically displayed increased expression of genes involved in transcription and protein processing, which probably reflect an increased rate of *de novo* production of MC-associated proteins (e.g. the CCL2 and CCL3 pro-inflammatory chemokines), due to the activating *KIT* mutation plus other microenvironmental and/or genetic changes.[23, 36] Overall, the distinct GEP displayed by indolent forms of the disease suggests that despite both ISMs- and ISMs+ BMMC carry the same *KIT* D816V mutation and show a similar overall activated immunophenotypic profile,[8] the response to this constitutive activation is different (activation of lipid metabolism *vs.* protein production), which could probably reflect the involvement of additional mechanisms modulating MC activation via Kit, FcεRI and/or HLA-II signalling, or a distinct genetic background among these patients. Altogether, these results support previous reports which propose the subclassification of these two groups of patients as clinically distinct subvariants of ISM.[4, 6]

Overall, BMMC from ASM patients also showed a unique GEP, clearly different from ISM (ISMs- and ISMs+) cases. Since the *KIT* D816V mutation was a common feature to all ASM and ISM cases analyzed, the heterogeneous clinico-biological features observed for ASM patients could be the result of e.g. coexistence of additional distinct genetic lesions. Altogether, our results point out the existence of significant deregulation of two distinct, but interconnected, cell functions in BMMC from ASM. Firstly, inhibition of apoptosis (reflected by the up-regulation of multiple anti-apoptotic genes, like the bifunctional apoptosis regulator *BFAR*) and secondly, cell cycle deregulation, associated with increased expression of genes involved in cell-cycle checkpoints like cyclin B1 (*CCNB1*), often described to be overexpressed in various human tumors[39,40,41] in association with an increased cell proliferation.[42] In line with these observations, although a slight increase in proliferation was observed for all variants of SM analyzed, only BMMC from ASM patients exhibited a high proliferation. Altogether, these findings suggest that different mechanisms could be involved in the pathogenesis of ASM, associated with either increased proliferation and/or inhibition of apoptosis, which would contribute to explain their heterogeneous clinco-biological behaviour[1, 2] and variable response to treatment.[43] In line with this hypothesis, *NRAS* and *TET2* mutations have been previously reported in a relatively small fraction of ASM patients.[44, 45] Further studies, in larger cohorts of ASM patients are required to confirm the existence of distinct GEP within ASM BMMC and their potential association with specific genetic lesions other than the *KIT* D816V mutation, as well as a distinct clinical behaviour of the disease and response to therapy.

Activation of Kit is known to also regulate adhesion of MC to the connective tissue matrix,[37, 38] as reflected here by deregulation of adhesion-related genes both in ASM and ISMs+. Nonetheless, distinct GEP were observed in aggressive versus indolent forms of the disease, since ASM BMMC displayed both a higher frequency of down-regulated transcripts and normal expression of other cell adhesion-associated genes up-regulated in ISMs+. Since one of the major features of ASM is frequent involvement of organs and tissues other than BM or the skin (e.g. spleen, liver, lymph nodes and/or peripheral blood), whereas presence of cutaneous lesions is a hallmark of ISMs+ patients,[1, 2] such distinct pattern of expression of adhesion-related genes could potentially contribute to explain the different patterns of organ/tissue involvement in both subtypes of SM.

Interestingly, despite the differences observed in the GEP of ISMs-, ISMs+ and ASM BMMC, a signature of 22 genes allowed for the classification of indolent versus aggressive SM; of note, such combination of genes could even discriminate BMMC of ASM from ISMs+ cases with multilineage BM involvement, a subgroup of ISM patients which has been previously described to exhibit similar immature immunophenotypical features of BMMC to those of ASM cases.[46] Since progression of ISMs+ cases with multilineage BM involvement by the *KIT* D816V mutation to ASM is not a rare event,[47] this gene signature could serve as prior knowledge for the discovery of biomarker candidates which would allow for monitoring patients with higher risk of progression (e.g. quantification of serum CCL23, IL1β or IL13 levels, up-regulated in ASM *versus* ISM with multilineage BM involvement by the *KIT* mutation). Further prospective studies are necessary in this regard.

A major concern regards the functional impact of GEP studies on protein expression. Overall, here we found a significant positive correlation between mRNA and protein levels for all genes tested; the only exception was *FCER1A,* for which an inverse correlation was observed (see Results section in the Online Repository). However, these results are consistent with previous reports on the metabolic rate of FcεRI of basophil, which suggest that FcεRI production is modulated during cell maturation and that once the cell is mature and exhibits high expression of surface membrane FcεRI, it slows down the rate of synthesis of the receptor.[48] In addition, expression of FcεRI on the cell surface is not only the result of the expression of FcεRIα, but it also depends on the rate of synthesis of FcεRIβ and FcεRIγ, as well as on the levels of free IgE.[48]

In summary, here we show for the first time that the D816V activating *KIT* mutation confers a common GEP to BMMC from distinct subvariants of SM; such common GEP is characterized by up-regulation of genes involved in innate and inflammatory immune responses, including IFN-induced genes involved in anti-viral responses. In addition, a distinct and unique GEP was also observed for each of the three subtypes of SM analyzed, which reflect an increased lipid *vs.* protein metabolism in ISMs- and ISMs+, together with an inhibition of apoptosis and/or an altered cell cycle regulation in ASM patients, highlighting the potential nature of relevant secondary genetic lesions and microenvironmental alterations specific for these subgroups of SM.

Authorship Contributions: C. T. performed experiments, analyzed/interpreted results, made the figures and wrote the paper; AC. G-M. Supervised the study, analyzed/interpreted results and critically reviewed the paper; M. J-A performed the detection of *KIT* mutation experiments, analyzed/interpreted results and critically reviewed the paper; L. S-M. contributed with technical support and critically reviewed the paper; C. P. performed statistical analysis of the data and critically reviewed the paper; I. A-T and A.M. performed the clinical follow-up of the patients and critically reviewed the paper; S. M. analyzed/interpreted the cell cycle experiments and critically reviewed the paper; JM. M contributed with technical support and critically reviewed the paper; P.B. performed the cell sorting of the distinct cellular subpopulations and critically reviewed the paper; L.E. supervised the study, performed clinical follow-up of the patients and critically reviewed the paper; A. O. designed the research, supervised the study and wrote the paper.

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Table I. Clinical and biological characteristics of systemic mastocytosis (SM) patients (n=26) and adult controls (n=5) in which the gene expression profile of bone marrow mast cells was analyzed by c-DNA microarrays.

CASE ID	DIAGNOSTIC GROUP	GENDER (M/F)	AGE (years)	% OF BMMC BY FLOW CYTOMETRY	PATTERN OF INVOLVEMENT OF BM CELLS BY THE *KIT* D816V MUTATION
1	Control BM	F	42	0.046	Non-mutated
2	Control BM	M	41	0.003	Non-mutated
3	Control BM	F	71	0.006	Non-mutated
4	Control BM	F	59	0.06	Non-mutated
5	Control BM	M	71	0.21	Non-mutated
6	ISM[s-]	F	63	0.29	Restricted to MC
7	ISM[s-]	F	28	0.12	Restricted to MC
8	ISM[s-]	M	58	0.21	Restricted to MC
9	ISM[s+]	M	46	1.0	Restricted to MC
10	ISM[s+]	F	54	0.52	Restricted to MC
11	ISM[s+]	M	24	0.89	Restricted to MC
12	ISM[s+]-AHNMD*	M	61	0.13	Restricted to MC
13	ISM[s+]	M	51	0.43	Myeloid lineages
14	ISM[s+]	M	32	0.31	Myeloid lineages
15	ISM[s+]	M	31	0.52	Myeloid lineages
16	ISM[s+]	M	61	4.9	Myeloid lineages
17	ISM[s+]	M	62	2.8	Myeloid plus lymphoid lineages
18	ISM[s+]	F	47	0.39	Myeloid plus lymphoid lineages
19	ISM[s+]	F	64	0.28	Myeloid plus lymphoid lineages
20	ASM-AHNMD[†]	M	64	0.13	Myeloid lineages
21	ASM	M	46	2.6	Myeloid lineages
22	ASM	M	72	1.8	Myeloid lineages
23	ASM	F	76	18.0	Myeloid lineages
24	ASM	M	64	2.9	Myeloid lineages
25	ASM	M	69	0.13	Myeloid lineages
26	ASM-AHNMD[‡]	F	55	18.0	Myeloid lineages
27	ASM	M	41	1.3	Myeloid plus lymphoid lineages
28	ASM	M	75	0.86	Myeloid plus lymphoid lineages
29	ASM	M	58	1.9	Myeloid plus lymphoid lineages
30	ASM	M	67	0.08	Myeloid plus lymphoid lineages
31	ASM-AHNMD[§]	M	82	5.3	Myeloid plus lymphoid lineages

M, male; F, female; MC, mast cells; BM, bone marrow; ISM[s-], indolent systemic mastocytosis without skin lesion; ISM[s+], indolent systemic mastocytosis with skin lesion; ASM, aggressive systemic mastocytosis; AHNMD, associated clonal non-MC lineage hematopoietic disease; *Myelodysplastic/myeloproliferative disorder; [†]Chronic myelomonocytic leukemia; [‡]Acute myeloid leukemia; [§]Myelodysplastic/myeloproliferative disorder and multiple myeloma.

Table II. Common gene expression profile of SM patients (ISMs-, ISMs+ and ASM): KEGG pathways significantly enriched for those genes (n=286) found to be differentially expressed in SM versus normal/reactive BMMC.

KEGG pathway code	Functional category	N. of genes differentially expressed/ N. of genes annotated to term (%)	Corrected p value	Gene ID
4640	**Hematopoietic cell lineage**	6/81 (7.4%)	0.005	*IL1R1, IL2RA, CR2, CD33, CD4, ITGA2B*
4610	**Complement and coagulation cascades**	4/64 (6.3%)	0.04	*CR2, SERPING1, CFH, PROS1*
4630	**Jak-STAT signaling pathway**	7/149 (4.7%)	0.02	*BCL2L1, IL2RA, LIF, PIM1, JAK3, PIK3R3, SOCS2*
4144	**Endocytosis**	7/192 (3.6%)	0.03	*STAMBP, RAB5B, IL2RA, DAB2, PML, CHMP5, ZFYVE9*
4142	**Lysosome**	8/118 (6.8%)	0.001	*NAGA, CTSD, CTSW, LIPA, CLN5, CTNS, GLB1, ATP6AP1*
4623	**Cytosolic DNA-sensing pathway**	4/56 (7.1%)	0.03	*CASP1, DDX58, POLR2H, POLR3B*
4210	**Apoptosis**	5/85 (9.4%)	0.03	*BCL2L1, IL1R1, CASP10, ENDOD1, PIK3R3*

SM, systemic mastocytosis; ISMs-, indolent SM without skin lesion; ISMs+, indolent SM with skin lesion; ASM, Aggressive SM; KEGG, Kyoto Encyclopedia of Genes and Genomes;

Table III. Functional profile of differentially expressed genes (up- or down-regulated) within the gene expression profile common to all diagnostic groups of SM analyzed: ISMs-, ISMs+ and ASM (n=286 genes).

Gene Ontology (GO) code	Functional category	N. of genes	Gene %*	Corrected p value	Gene ID
0007165	Signal transduction	24	2.0%	0.02	LGALS3BP, CD33, SIGLEC8, MX1, GRB10, ITPK1, GDI1, ICK, MAPKAPK3, CD4, CCL3, AKAP12, SHISA5, NEK6, ARHGAP1, IL1R1, NR4A1, SNX17, CASP1, CCL23, PDE3A, TYROBP, PRMT2, **EPS8**
0006955	Immune response	15	4.7%	<0.0001	CR2, OAS3, LIF, CST7, IL2RA, OAS1, CD4, CCL3, ENPP2, OAS2, CTSW, PDCD1LG2, IL1R1, CCL23, MR1
0006810	Transport	15	2.5%	0.02	APOL2, SLC35A1, TXNRD3, UQCRQ, SLC25A30, TMED8, NDUFS5, CTNS, APOL1, FRRS1, CROT, PCTP, FADS1, DYNLRB1, NDUFS2
0019221	Cytokine-mediated signaling pathway	13	8.8%	<0.0001	XAF1, OAS3, IRF2, MX1, GBP1, OAS1, MX2, JAK3, USP18, OAS2, IFIT3, PML, IFIT1
0045087	Innate immune response	13	4.8%	0.0004	CR2, APOL1, BCL2L1, CLU, DDX58, JAK3, MAPKAPK3, SERPING1, IL1R1, CASP1, UBA7, CASP10, TRIM25
00060337	Type I interferon-mediated signaling pathway	10	17.0%	<0.0001	XAF1, OAS3, IRF2, MX1, OAS1 ,MX2, USP18, OAS2, IFIT3 ,IFIT1
0009615	Response to virus	9	6.5%	0.001	MX1, MX2, CLU, IFI44L, ZNF175, POLR3B, PML, IFIT1, TRIM25
0006917	Induction of apoptosis	8	4.4%	0.02	MX1, ZMAT3, SHISA5, NR4A1, PML, ERCC3, PRMT2, CASP10
0006886	Intracellular protein transport	8	4.3%	0.02	STON2, MLPH, ARFIP1, VIPAR, NAPA, SNX17,KDELR1,GPR34
0055114	Oxidation-reduction process	8	3.8%	0.03	C10orf58, IDH1, ACOX1, MTHFD1, FADS1, TBXAS1, OGDH, NDUFS2
0006091	Generation of precursor metabolites and energy	6	10.7%	0.002	ACOX1, PHKA1, FECH, CROT, COX17, OGDH
0060333	Interferon-gamma-mediated signaling pathway	6	10.3%	0.002	OAS3, IRF2, GBP1, OAS1, OAS2, PML
0006368	Transcription elongation from RNA polymerase II promoter	5	8.0%	0.02	GTF2A1, GTF2H1, ERCC3, ELP3, POLR2H
0006605	Protein targeting	4	12.9%	0.01	STXBP4, MLPH, AKAP12, PML
0045071	Negative regulation of viral genome replication	3	18.8%	0.02	OAS3, OAS1, IFIT1
0006164	Purine nucleotide biosynthetic process	3	16.7%	0.02	OAS1, MTHFD1, OAS2
0006362	Transcription elongation from RNA polymerase I promoter	3	16.7%	0.02	GTF2H1, ERCC3, POLR2H
0006363	Termination of RNA polymerase I transcription	3	15.0%	0.03	GTF2H ,ERCC3, POLR2H
0006360	Transcription from RNA polymerase I promoter	3	13.0%	0.04	GTF2H1, ERCC3, POLR2H
0006361	Transcription initiation from RNA polymerase I promoter	3	13.0%	0.04	GTF2H1, ERCC3, POLR2H
0060700	Regulation of ribonuclease activity	2	100.0%	0.008	OAS3,OAS1
0035457	Cellular response to interferon-alpha	2	50.0%	0.02	OAS1, IFIT3
0021860	Pyramidal neuron development	2	40.0%	0.03	UQCRQ, OGDH
0007184	SMAD protein import into nucleus	2	33.3%	0.04	ZFYVE9, PML

SM, systemic mastocytosis; ISMs-, indolent SM without skin lesion; ISMs+, indolent SM with skin lesion; ASM, aggressive SM; BM, bone marrow; GO, Gene Ontology.

Down- regulated genes are shown in bold and underlined, whereas up-regulated genes are not highlighted or underlined.

*Percentage of genes within a functional category is given as the ratio between the number of genes differentially expressed in the gene expression profile common to all diagnostic subtypes of SM assigned to a GO term and the total number of genes included in the array which are annotated to the same GO term.

Table IV. Functional profile of those genes differentially expressed (up- or down-regulated) by BMMC from patients with indolent SM with (ISMs+) or without (ISMs-) skin lesions.

Gene group	Gene Ontology (GO) code	Functional category	N. of genes	Gene %*	Corrected p value	Gene ID
ISMs- (n=474 genes)	0006468	**Protein phosphorylation**	15	3.9%	0.04	*ILK, ERN1, TESK2, CSK, CDK4, GOLGA5, JAK3, ICK, NEK6, GTF2H1, ERCC3, PIM1, TXK, CAMK2G, POLR2H*
	0006629	**Lipid metabolic process**	12	4.9%	0.03	*APOL2, ACOX1, LIPA, APOL4, CLU, PAFAH2, TECR, FADS1, PDE3A, LPIN1, ACER2, PNPLA6*
	0007166	**Cell surface receptor linked signaling pathway**	10	6.2%	0.02	*CABIN1, P2RX7, IL2RA, CD2, TNFRSF14, CD4, MYD88, LY6E, IL1R1, FCER1A, **CHRM4***
	0044255	**Cellular lipid metabolic process**	9	7.1%	0.02	*IDH1, RGL1, ACOX1, SLC25A20, CROT, TECR, FADS1, INPPL1, LPIN1*
	0031295	**T cell costimulation**	7	11.1%	0.007	*HLA-DRB1, CSK, TNFRSF14, CD4, PIK3R3, PDCD1LG2, HLA-DRB5*
	0035335	**Peptidyl-tyrosine dephosphorylation**	6	10.3%	0.02	*PTPN5, CSK, PTPN9, PDCD1LG2, DUSP3, EYA3*
	0050852	**T cell receptor signaling pathway**	6	8.1%	0.05	*HLA-DRB1, CSK, CD4, HLA-DRB5, TXK, LAT*
	0032480	**Negative regulation of type I interferon production**	4	13.3%	0.05	*UBE2L6, DDX58, UBA7, TRIM25*
	0050868	**Negative regulation of T cell activation**	3	33.3%	0.02	*GIMAP5, JAK3, DUSP3*
	0006290	**Pyrimidine dimer repair**	2	66.7%	0.04	*POLH, DDB2*
	0032957	**Inositol trisphosphate metabolic process**	2	66.7%	0.04	*ITPK1, INPPL1*
ISMs+ (n=438 genes)	0007155	**Cell adhesion**	19	3.7%	0.01	*LGALS3BP, CD33, NRP1, SIGLEC8, COL12A1, CD300A, SELPLG, MPZL2, CLSTN1, PERP, CD4, ITGA2B, FERMT3, CDH12, FREM1, DST, INPPL1, CCL2, **CELSR1***
	0006915	**Apoptosis**	18	3.1%	0.05	*FASTKD1, ERN1, XAF1, NMT1, PSME1, NCSTN, IL2RA, CSRNP2, BCL2L1, ZMAT3, PERP, SHISA5, NEK6, CIDEB, CASP1, TNFRSF10B, CASP10, **HCAR2***
	0006468	**Protein phosphorylation**	17	4.5%	0.005	*RNASEL, ABL2, ILK, ERN1, TESK2, CSK, GOLGA5, JAK3, ICK, NEK6, GTF2H1, ERCC3, PIM1, CCL2, TXK, CAMK2G, POLR2H*
	0006464	**Protein modification process**	9	6.1%	0.01	*ABL2, SLC35A1, UEVLD, ST8SIA4, ADPRH, RIMKLB, PADI2, UBA7, HERC6*
	0007166	**Cell surface receptor linked signaling pathway**	9	5.6%	0.02	*CABIN1, IL2RA, TNFRSF14, CD4, LY6E, IL1R1, FCER1A, CCL2, TNFRSF10B*
	0006457	**Protein folding**	9	5.1%	0.03	*EDEM2, PPIB, DNAJB9, AARS, FKBP15, AHSA1, MPDU1, MESDC2, MLEC*
	0006367	**Transcription initiation from RNA polymerase II promoter**	6	9.2%	0.01	*GTF2A1, GTF2E1, GTF2H1, POLR2K, ERCC3, POLR2H*
	0051607	**Defense response to virus**	5	11.1%	0.02	*RNASEL, OAS3, OAS1, RSAD2, UNC13D*
	0035335	**Peptidyl-tyrosine dephosphorylation**	5	8.6%	0.04	*PTPN5, CSK, PTPN9, PDCD1LG2, EYA3*
	0031295	**T cell costimulation**	5	7.9%	0.05	*CSK, TNFRSF14, CD4, PIK3R3, PDCD1LG2*
	0006370	**mRNA capping**	4	14.2%	0.02	*GTF2H1, POLR2K, ERCC3, POLR2H*
	0006383	**Transcription from RNA polymerase III promoter**	4	11.4%	0.04	*POLR3B, POLR2K, GTF3C3, POLR2H*
	0045176	**Apical protein localization**	3	30.0%	0.02	*NAPA, GOPC, **CELSR1***
	0006385	**Transcription elongation from RNA polymerase III promoter**	3	25.0%	0.02	*POLR3B, POLR2K, POLR2H*
	0006386	**Termination of RNA polymerase III transcription**	3	25.0%	0.02	*POLR3B, POLR2K, POLR2H*
	0030100	**Regulation of endocytosis**	3	20.0%	0.03	*ABL2, STON2, SNX17*
	0030099	**Myeloid cell differentiation**	3	16.7%	0.05	*RUNX1, PML, RASGRP4*
	0019853	**L-ascorbic acid biosynthetic process**	2	100.0%	0.01	*AKR1A1, GSTO1*
	0006290	**Pyrimidine dimer repair**	2	66.7%	0.02	*POLH, DDB2*
	0032957	**Inositol trisphosphate metabolic process**	2	66.7%	0.02	*ITPK1, INPPL1*

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BM, bone marrow; MC, mast cell; SM, systemic mastocytosis; ISMs-, indolent SM without skin lesion; ISMs+, indolent SM with skin lesion; GO, Gene Ontology.

Down- regulated genes are shown in bold and underlined, whereas up-regulated genes are not highlighted nor underlined.

*Percentage of genes within a functional category is given as the ratio between the number of genes differentially expressed in BMMC from a specific subtype of ISM (ISMs- or ISMs-) assigned to a GO term and the total number of genes included in the array which are annotated to the same GO term.

Table V. Functional profile of those genes differentially expressed (up- or down-regulated) by BMMC from patients with ASM (n=758).

Gene Ontology (GO) code	Functional category	N. of genes	Gene %*	Corrected p value	Gene ID
0006915	**Apoptosis**	34	5.8%	0.001	*ZMAT3, OSM, TMEM85, PSMD10, NET1, BCL2L1, PSME1, NCSTN, CIDEB, TPX2, PSMB3, TCTN3, IL1B, NEK6, PSMF1, IL2RA, GADD45A, CASP1, NMT1, EAF2, DYNLL1, NLRP2, STK3, DAP3, CASP10, CSRNP2, H1F0, SHISA5, XAF1, BFAR, TNFRSF10B, FASTKD1, ZC3H12A,* **_HCAR2_**
0007155	**Cell adhesion**	26	5.0%	0.04	*LGALS3BP, CD33, NRP1, SIGLEC8, AMIGO2, CCR1, CD300A, SELPLG, MPZL2, F11R, CLSTN1, SIGLEC10, CD4, ITGA2B, FERMT3, EMR1, MPZL3, FREM1, ITGB7, DST, CCL2,* **_CELSR1_**, **_DCHS1_**, **_HAPLN4_,_** **_NID1_**, **_SDK2_**
0000278	**Mitotic cell cycle**	22	7.2%	0.002	*POLD3, CCNA1, PSMB3, MIS12, ANAPC7, RFC3, SKP2, PSME1, PSMF1, CDK4, DCTN2, ZWILCH, KIF18A, DYNC1I2, PSMD10, CDC6, MCM7, CDC23, CCNB1, DYNLL1, PRIM1, ORC5L*
0006916	**Anti-apoptosis**	14	7.0%	0.04	*STAMBP, AMIGO2, IL1B, CEBPB, BCL2L1, CLU, MYD88, PAFAH2, BECN1, SOCS2, HTATIP2, CCL2, BFAR,* **_NANOS3_**
0000084	**S phase of mitotic cell cycle**	12	10.8%	0.003	*POLD3, PSMB3, RFC3, SKP2, PSME1, PSMF1, CDK4, PSMD10, CDC6, MCM7, PRIM1*
0000075	**Cell cycle checkpoint**	12	9.3%	0.01	*PSMF1, ANAPC7, ORC5L, RFC3, CDC23, ERCC3, PSMB3, CCNB1, PSMD10, MCM7, PSME1, CDC6*
0000082	**G1/S transition of mitotic cell cycle**	12	8.5%	0.02	*PSMF1, ORC5L, SKP2, PSMB3, CCNB1, CCNA1, PSMD10, MCM7, CDK4, PSME1, CDC6, PRIM1*
0007166	**Cell surface receptor linked signaling pathway**	12	7.4%	0.03	*CABIN1, CCR1, IL2RA, CD4, MYD88, LY6E, IL1R1, FCER1A, CCL2, BRD8,TNFRSF10B,* **_GFRA1_**
0006464	**Protein modification process**	11	7.5%	0.04	*ABL2, SLC35A1, UEVLD, ST8SIA4, ST3GAL1, ADPRH, RIMKLB, LCMT1, PADI2, UBA7, HERC6*
0071260	**Cellular response to mechanical stimulus**	9	18.4%	0.001	*IL13, IL1B, GADD45A, MYD88, CASP8AP2, TLR3, CASP1, MAP2K4, TNFRSF10B*
0000216	**M/G1 transition of mitotic cell cycle**	8	10.3%	0.03	*ORC5L, PSMB3, PSME1, PSMF1, PSMD10, CDC6, MCM7, PRIM1*
0031145	**Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process**	8	10.1%	0.03	*PSMB3, ANAPC7, SKP2, PSME1, PSMF1, PSMD10, CDC23, CCNB1*
0000079	**Regulation of cyclin-dependent protein kinase activity**	7	12.7%	0.04	*BLM, CCNA1, CKS2, GADD45A, GTF2H1, CDC6, CCNB1*
0006289	**Nucleotide-excision repair**	7	10.6%	0.04	*POLD3, RFC3, GTF2H5, GTF2H1, POLR2K, ERCC3, POLR2H*
0006283	**Transcription-coupled nucleotide-excision repair**	6	14.0%	0.04	*POLD3, RFC3, GTF2H1, POLR2K, ERCC3, POLR2H*
0051607	**Defense response to virus**	6	13.3%	0.04	*RNASEL, OAS3, NLRP3, OAS1 ,TLR3, RSAD2*
0008652	**Cellular amino acid biosynthetic process**	5	14.7%	0.05	*ASNSD1, ALDH18A1, ENOPH1, MTHFD1,* **_GPT_**
0009311	**Oligosaccharide metabolic process**	4	33.3%	0.02	*MGAT2, ST8SIA4, ST8SIA6, NAGA*
0071310	**Cellular response to organic substance**	3	42.9%	0.04	*IL1B, CASP1,* **_TRIM71_**
0045080	**Positive regulation of chemokine biosynthetic process**	3	37.5%	0.04	*IL1B, MYD88, TLR3*
0000731	**DNA synthesis involved in DNA repair**	3	33.3%	0.04	*POLD3, RFC3, POLH*
0019853	**L-ascorbic acid biosynthetic process**	2	100.0%	0.03	*AKR1A1, GSTO1*
0034638	**Phosphatidylcholine catabolic process**	2	100.0%	0.03	*LIPC, ENPP2*
0035860	**Glial cell-derived neurotrophic factor receptor signaling pathway**	2	100.0%	0.03	**_GFRA1_**, **_GFRA2_**
0042506	**Tyrosine phosphorylation of Stat5 protein**	2	100.0%	0.03	*JAK3, OSM*

BM, bone marrow; MC, mast cell; SM, systemic mastocytosis; ASM, aggressive SM; GO, Gene Ontology.

Down- regulated genes are shown in bold and underlined, whereas up-regulated genes are not highlighted nor underlined.

*Percentage of genes within a functional category is given as the ratio between the number of genes differentially expressed in BMMC from ASM patients which are assigned to a GO term, and the total number of genes included in the array which are annotated to the same GO term.

<u>**FIGURE LEGENDS:**</u>

Figure 1. Venn diagram showing the overlap between genes differentially expressed by BMMC from ISMs- (continuous-line), ISMs+ (dashed line) and ASM (dotted line) patients vs. normal/reactive BMMC. Numbers inside each compartment represent the number of genes showing a significantly different (FDR<1% and fold change ≥|2.0|) expression in SM vs. normal/reactive BMMC. ISMs-, indolent systemic mastocytosis without skin lesion; ISMs+, ISM with skin lesions; ASM, aggressive SM.

Figure 2. Altered GEP common to distinct subtypes of SM (ISMs-, ISMs+ and ASM): network pathway analysis of those genes which are associated with the immune response and/or have been previously described to be regulated or interact with *KIT.* Up-regulated genes are depicted in red, the colour intensity reflecting the fold-change levels versus those observed in normal/reactive BMMC. Relationship between genes: i) activation (lines ending in arrows), ii) inhibition (lines ending in a perpendicular line), iii) binding (lines joining two genes); iiii) direct action (solid lines) and, iiiii) indirect effects (dashed lines). Lines reflecting interactions with *KIT* are highlighted in black. The different symbols represent distinct gene functions:◯ = transcription factor, ◖◗ = transmembrane receptor, ◇ = enzyme,◇ = peptidase activity; △ = transporter, ▽ = kinase activity, ▢ = cytokine, ◯ = other. * - Preferential localization in the cell is not known.

Figure 3. Genes differentially expressed by BMMC from ISM vs ASM patients. Expression patterns are summarized in the column in the left - "gene expression levels (GEP)" - where those genes which were differently expressed (FDR<1% and absolute fold change ≥ 2.0) in SM patients versus normal/reactive BMMC and that contributed to the discrimination between ISM and ASM are shown by color codes (white squares: expression not significantly different from normal/reactive BMMC, and; grey squares: genes significantly up-regulated in specific disease groups; the grey intensity reflects increasing fold change values). Centroid plots in the middle column show a ranked list of the same 22 genes that better discriminated between the two groups of patients. Gene expression charts on the right, display median values and the 95%

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confidence interval log-transformed expression levels for each individual gene in normal/reactive BMMC (n=5), ISMs- (n=3), ISMs+ with *KIT* mutation restricted to BM MC (n=4), ISMs+ with multilineage *KIT* mutation (n=7) and ASM (n=12) BMMC.

ISM, indolent systemic mastocytosis; ISMs-, ISM without skin lesion; ISMs+, ISM with skin lesion; ASM, aggressive SM; AU, log-transformed fluorescence arbitrary units; N/R BMMC, normal/reactive bone marrow mast cells; MCR, mast cell restricted *KIT* D816V mutation; ML, multilineage (myeloid or myeloid plus lymphoid) involvement by the *KIT* D816V mutation.
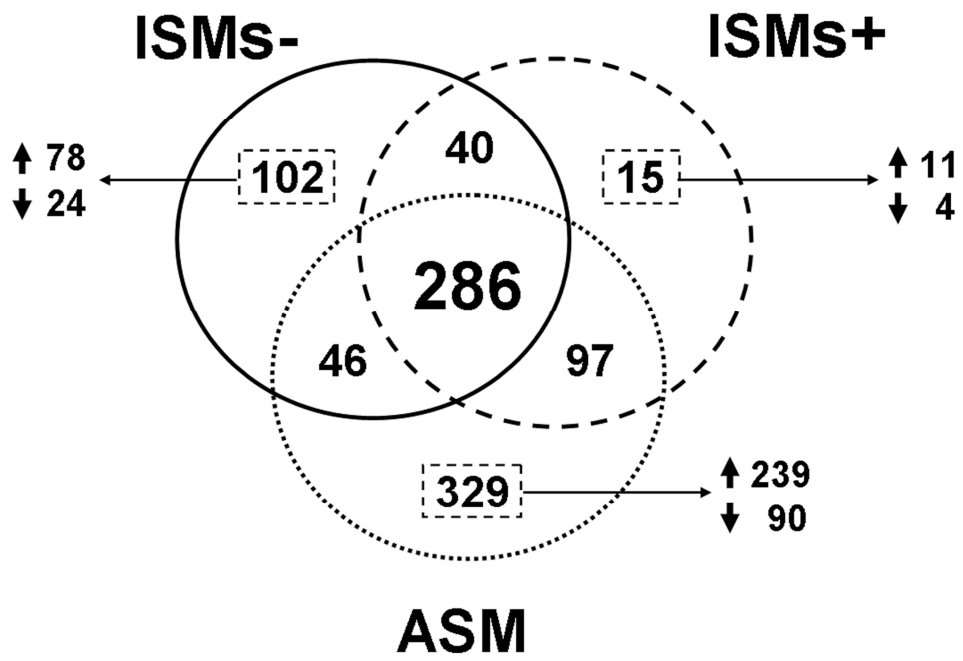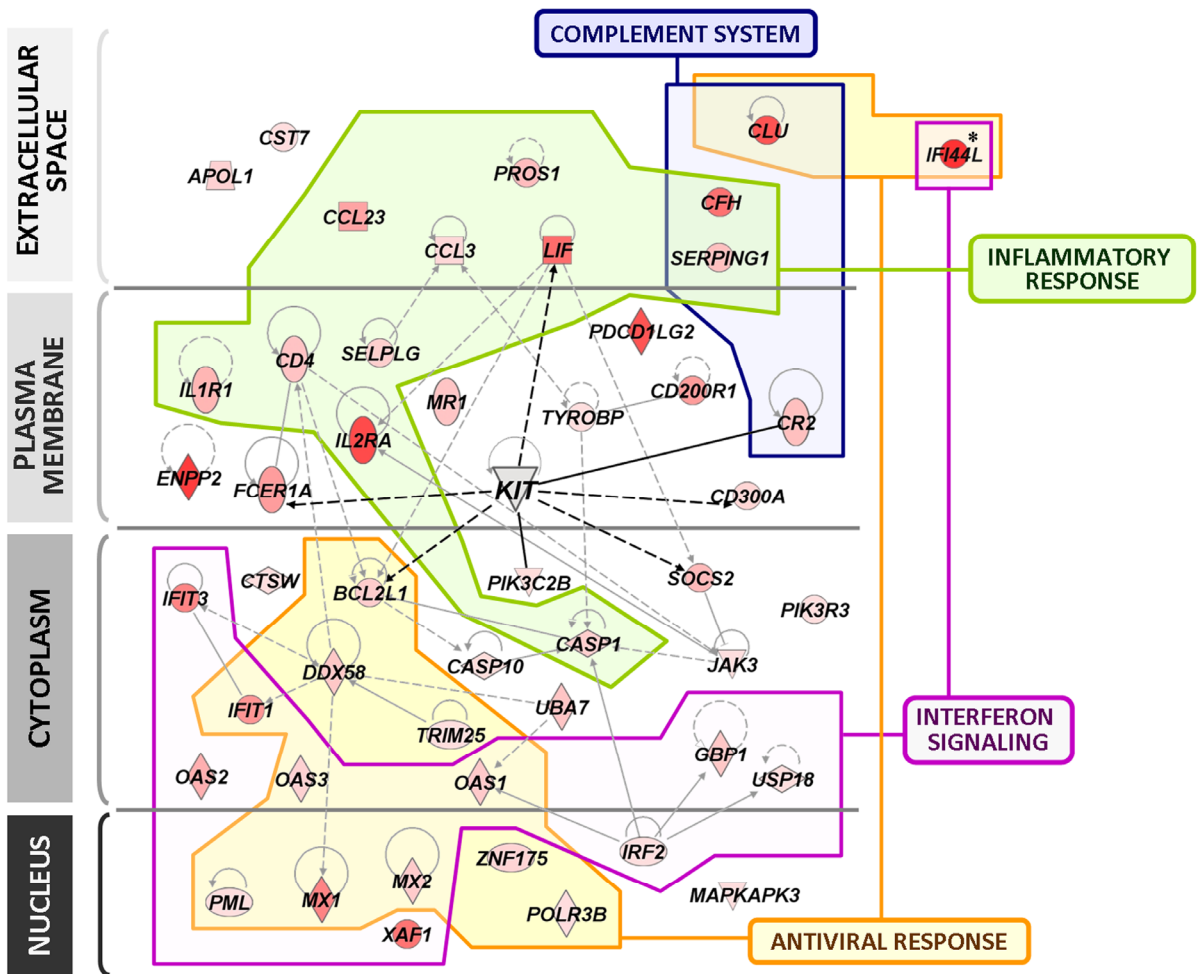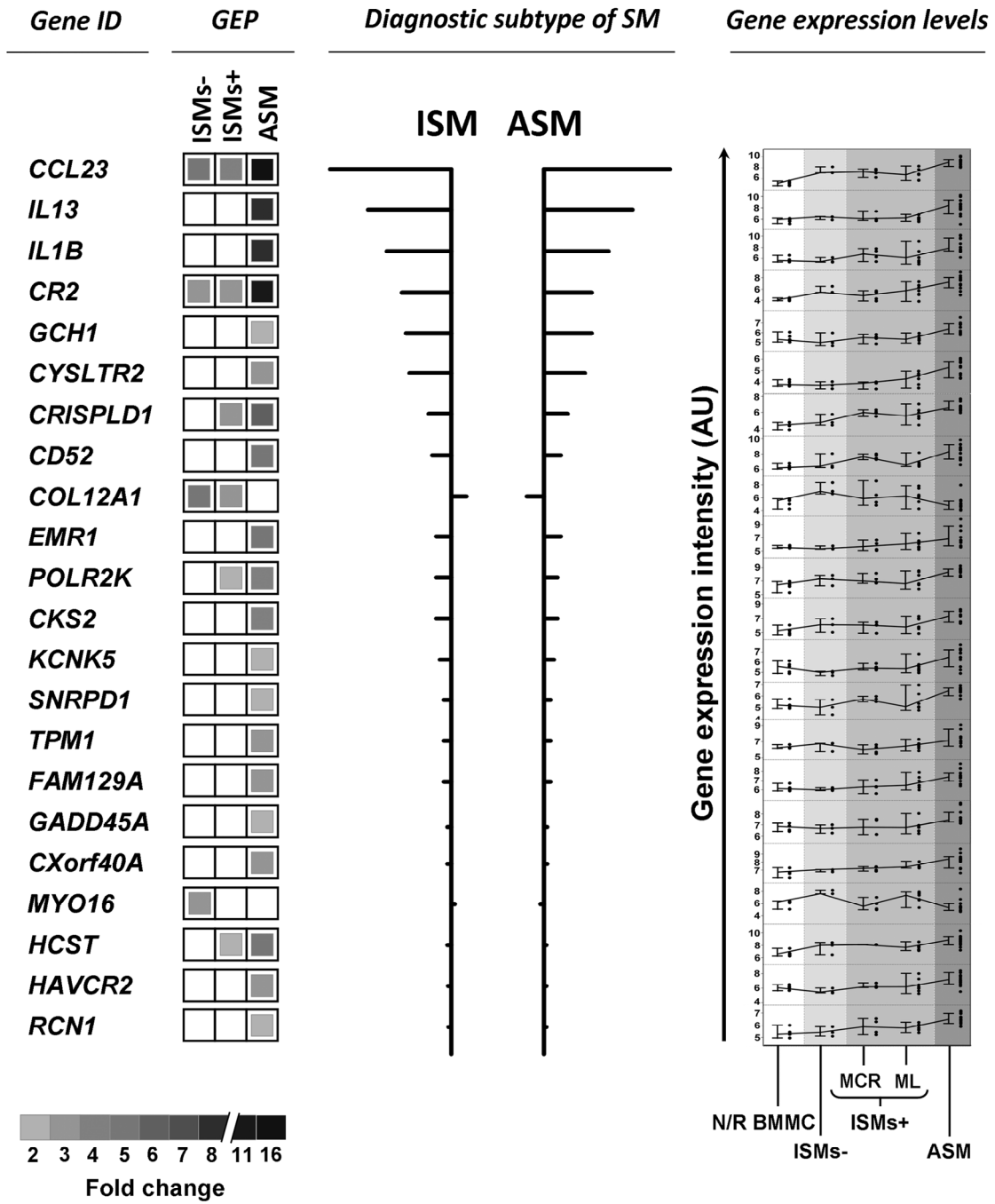
Figure 1.

Figure 2.

Figure 3.

MATERIAL AND METHODS

Isolation of MC and other subpopulations of BM nucleated cells, DNA extraction and detection of the D816V _KIT_ mutation. Isolation of mast cells (MC) and other subpopulations of nucleated bone marrow (BM) cells was performed within the first 24h after sample collection, using a four-way fluorescence-activated cell sorter (FACSAria, Becton Dickinson Biosciences-BD-, San Jose, CA) equipped with the FACSDiVa software (BD), as previously described.[E1] Briefly, prior to sorting, cells were stained with a five-color combination of monoclonal antibodies (MAb) – CD3/CD45/CD117/CD34/CD14 conjugated with pacific blue (PacB)/ fluorescein isothiocyanate (FITC) / phycoerythrin (PE)-cyanin 7 (Cy7) / allophycocyanin (APC) / APC-H7 -, using well established stain-and-then-lyse-and-wash procedures described elsewhere.[E1] Isolation of BMMC, eosinophils, monocytes, neutrophils, CD34+ hematopoietic precursor cells (HPC) and lymphocytes (purity ≥97%) was performed according to well-established phenotypic criteria previously described in detail.[E1]

Genomic DNA was extracted using the GenElute Mammalian Genomic DNA Miniprep, (Sigma-Aldrich, St Louis, MO, USA), NucleoSpin® Tissue XS (Macherey-Nagel, Düren, Germany) or the REDExtract-N-AmpTM blood PCR kit (Sigma-Aldrich), according to the instructions of the manufacturers. Positivity for the D816V _KIT_ mutation was determined using a PNA-mediated PCR-clamping technique (LightCycler thermocycler; software version 3.5; Roche Diagnostics GmbH, Mannheim, Germany) and the DNA Master Hybridization probes Kit (Roche Diagnostics), followed by sequencing of the PCR products in both directions, using the 5'- CAG CCA GAA ATA TCC TCC TTA CT-3' and 5'-TTG CAG GAC TGT CAA GCA GAG-3' primers (Isogen Life Sciences, Maarsen, The Netherlands), as described elsewhere.[E1, E2]

RNA extraction and microarray hybridization. Analysis of GEP was performed on RNA extracted from purified BMMC from 31 individuals, including 5 controls (3 healthy donors and 2 individuals carrying a B-cell lymphoproliferative disorder which exhibited reactive mast cell hyperplasia – cases #4 and #5) and 26 SM patients (Table I). For each sample, total RNA was extracted from highly pure (≥97%) BMMC using either the TRizol® Reagent (Invitrogen,

Carlsbad, CA) or the NucleoSpin® RNA XS kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Purity and integrity of the RNA were assessed for each sample, using an Agilent 2100 Bioanalyzer with either the RNA 6000 Nanochips or Picochips (Agilent Technologies, Santa Clara, CA, USA). GEP were determined using the Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) employing the hybridization, washing and laser scanning procedures recommended by the manufacturer.

Multiparameter flow cytometry immunophenotypic studies and cell cycle analyses.
Multiparameter flow cytometry (MFC) immunophenotypic studies were used to validate microarray results for a set of 10 genes (*CD2, CD4, CD33, CD52, FCER1A, HLA-DRB1, HLA-DRB5, IL2RA, IL3RA* and *LAIR1)* (Supplemental Table E1) which code for proteins primary located at the cell surface membrane and which were not previously described to be stored in BMMC granules. For these studies, BM aspirates from the same patients initially included in the microarray analyses (validation set #1) (Table I) and another independent group of patients (n=134) (validation set #2) were processed as previously described,[E3] following the recommendations of the REMA.[E4]

Cell cycle analyses. Assessment of the cell cycle distribution of BMMC (percentage of G_0/G_1 and S plus G_2/M phases) was performed by a previously described 3-color direct immunofluorescence stain-and-then-lyse-and-wash technique,[E5] in which nuclear DNA of single cells was stained with DRAQ5[TM] (Vitro SA, Madrid, Spain) and BMMC identified by their unique pattern of expression of CD45 and CD117 (Supplemental Table E1). The proliferation index (PI) (% of S plus G_2/M cells) was assessed by two different observers for those cases for which >100 BMMC could be analysed (n=29), using the INFINICYT™ software (Cytognos SL, Salamanca, Spain) and a well established strategy.[E5]

Statistical methods. For all continuous variables, their median, mean and standard deviation (SD), as well as range and both the 25th and 75th and the 10th and 90th percentiles, were calculated. For categorical variables, frequencies were used. Statistical significance (p value

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≤0.05 and FDR correction for multiple comparisons of <10%) was determined by the non-parametric Kruskal-Wallis and Mann-Whitney U tests; a lineal regression model was used to explore the degree of correlation between different variables. For all statistical analyses the SPSS 15.0 (SPSS, Chicago, IL, USA) and MATLAB R2010b (Mathworks, Natick, MA, USA) software programs were used.

RESULTS

Multiparameter flow cytometry imunophenotypic studies for validation of GEP microarray

data. Validation studies performed at the protein level with the same set of patients used for GEP microarray analyses showed the existence of a significant correlation between gene expression data (unlogged arbitrary intensity units) and protein expression - mean expression intensity expressed as mean fluorescence intensity (MFI) arbitrary units - for all 7 genes tested. These included the *IL2RA* (r^2=0.9, p<0.0001; n=10)*, CD4* (r^2=0.7, p=0.013; n=7)*, IL3RA* (r^2=0.5, p<0.0001; n=22), *CD2* (r^2=0.4, p=0.004; n=21)*, HLA-DRB1* and *HLA-DRB5* (r^2=0.7, p=0.004; n=11) genes, and the *FCER1A* gene for which an inverse correlation (r^2=-0.5, p=0.0003; n=19) was found. Furthermore, validation of the GEP in a second and larger cohort of patients (n=134) (validation set #2) showed overall similar profiles for gene and protein expression for CD25, LAIR1 (CD305), CD2, HLA-DR and CD52 (Supplemental Figure E1). Interestingly, despite *CD4, CD33* and *FCER1A* genes were up-regulated in the common GEP, significantly increased membrane expression of the CD4 and FcεRI was restricted to ISMs- patients (p≤0.0001), and significantly increased expression of the CD33 protein was only detected among ISM (p<0.0001) patients, ASM patients exhibiting either normal (5/7; 71%) or increased CD33 protein levels (2/7; 29%) (Supplemental Figure E1). Conversely, up-regulation of the *IL3RA* gene was exclusively observed among ASM while all subtypes of SM analysed displayed overall increased protein levels (p<0.0001), but at variable frequencies: 67% (14/21) ISMs-, 46% (22/48) ISMs+ and 100% (7/7) ASM patients, respectively.

Cell cycle analysis revealed an overall slight increased proliferation index (PI; % of *S* plus *G₂/M* cells) of BMMC among all three SM subtypes analyzed (p<0.02 *vs.* normal/reactive BMMC), although cases with high proliferation (PI >5%) were only found among ASM patients (2/5 cases; 40%) (Supplemental Figure E1).

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Supplemental Table E1: Antibody reagents used for the immunophenotypic and cell cycle analyses of bone marrow mast cells.

PURPOSE	GENE	AB CONJUGATE	CLONE	SOURCE
BM MC IDENTIFICATION	N/A	CD34- PerCP Cy5.5	8G12	BD Biosciences[1]
	N/A	CD45 - Pacific Orange	HI30	Invitrogen[2]
	N/A	CD45 - FITC	HI30	BD Biosciences[1]
	N/A	CD45 – PerCP Cy5.5	2D1	BD Biosciences[2]
	N/A	CD117- PE	YB5.B8	BD Biosciences[1]
	N/A	CD117- PE Cy7	104D2D1	Beckman Coulter[3]
	N/A	CD117 - APC	YB5.B8	BD Biosciences[1]
VALIDATION OF MICROARRAY RESULTS	*CD2*	CD2 - FITC	S5.2	BD Biosciences[1]
	CD4	CD4 – Pacific Blue	RPA-T4	BD Biosciences[1]
	CD33	CD33 – APC	P67,6	BD Biosciences[1]
	CD52	CD52 - PE	CF1D12	Invitrogen[2]
	FCER1A	FcεRI - FITC	Polyclonal	Invitrogen[2]
	HLA-DRB1 HLA-DRB5	HLA-DR – FITC	L234	BD Biosciences[1]
	IL2RA	CD25 - PE	2A3	BD Biosciences[1]
	IL3RA	CD123 - PE	9F5	BD Biosciences[1]
	LAIR1	CD305 – PE	DX26	BD Biosciences[1]

N/A- not applicable.

[1] Becton/Dickinson Biosciences (San José, CA, USA);
[2] Invitrogen (Carlsbad, CA, USA);
[3] Beckman-Coulter (Miami, FL, USA);

Supplemental Figure E1. Immunophenotypic characteristics and proliferation index (percentage of S plus G2/M cells) of bone marrow (BM) mast cells (MC) from patients with systemic mastocytosis compared to normal/reactive bone marrow MC (control). Panels A-I show the expression of individual markers as mean fluorescence intensity (MFI) values per MC. Panel J shows proliferation index of BMMC. ISM, indolent systemic mastocytosis; ISMs-, ISM without skin lesion; ISMs+, ISM with skin lesions; ASM, aggressive SM.

Supplemental Figure E1.
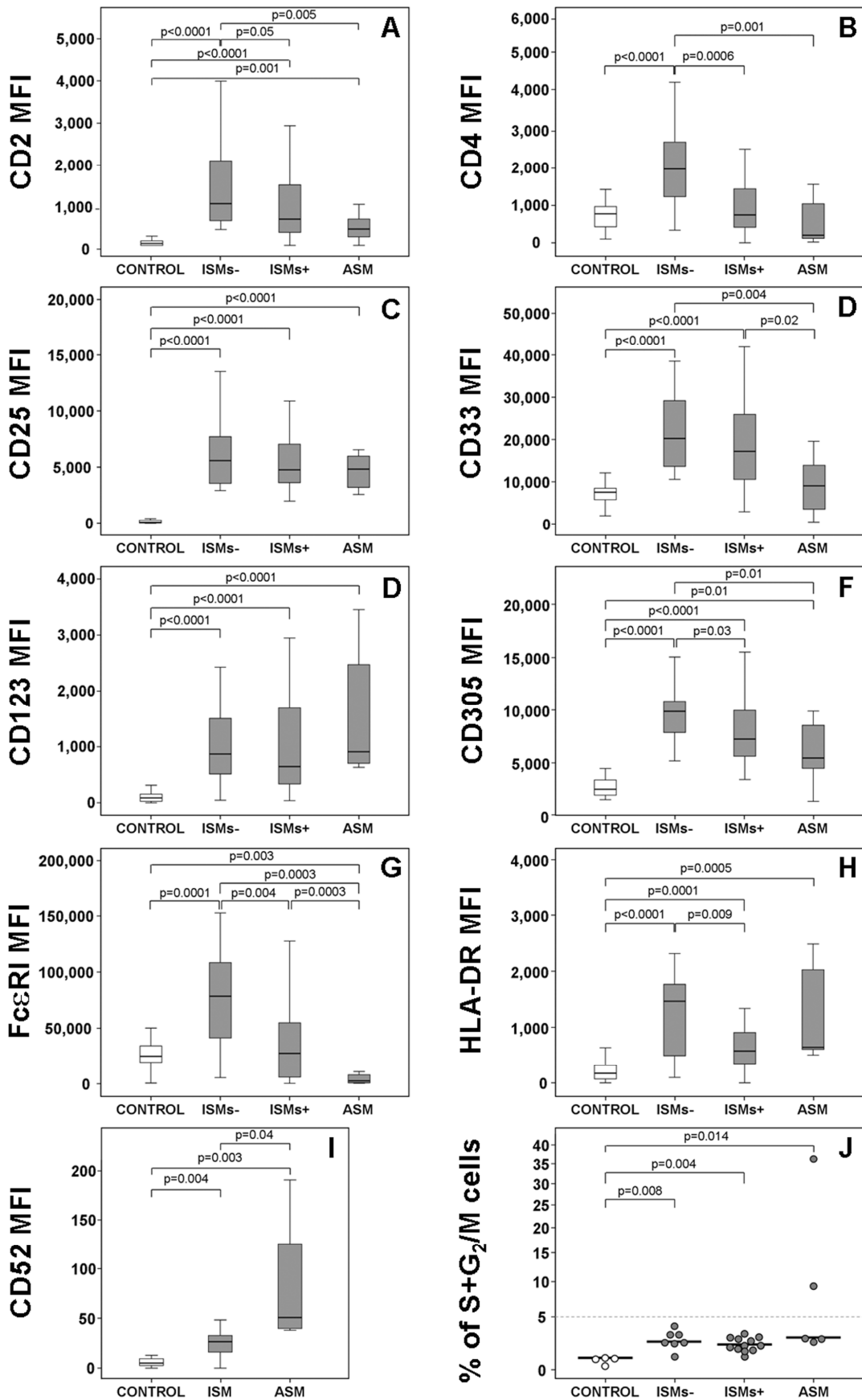
Chapter 4 |
GENERAL DISCUSSION

During the past few decades, increasing knowledge has accumulated about the biology of mast cells. This has contributed to a better understanding of the role these cells play in human physiology, as well as in the pathogenesis of multiple diseases, including clonal mast cell disorders [reviewed in [3-5,23]]. In this regard, identification of SCF as the main growth factor driving mast cell development and proliferation, set the basis for the discovery of the D816V *KIT* mutation, which is now recognized as the a hallmark of SM.[16,253,257]

Despite the great impact that activating *KIT* mutations have on the proliferation and survival, as well as on the morphology, immunophenotype and function of mast cells,[26] these mutations do not explain *per se* the highly variable and heterogeneous features that clonal mast cells display from patient to patient, from the histopathological, morphological immunophenotypic and molecular/genetic point of view. Standardization efforts made in the assessment of such features of the disease,[112,252,312,313] have set up the basic pillars for the definition of current diagnostic criteria of mastocytosis. As an example, the establishment of a well-defined strategy for the identification, enumeration and characterization of BM mast cells by MFC contributed to overcome several important limitations inherent to the study of rare BM cell populations, for which there was not a previously defined specific marker and phenotype.[110,112] As a consequence of such standardization efforts, several aberrant immunophenotypic features have been identified on clonal BM mast cells; in turn, these allowed for both a better understanding of the biological basis underlying altered mast cell functions (e.g. increased expression of complement regulator proteins like CD55 or CD59, which confer clonal mast cells an increased resistance to complement mediated cell lysis)[124] and the identification of new surrogate markers for mast cell clonality (e.g. CD25),[110,376] which are currently considered robust criteria for the diagnosis of SM.[359] Based on such advances, new diagnostic criteria for SM were progressively established.

The first comprehensive clinico-morphological classification of mastocytosis was proposed in 1991. Overall, it identified only two major categories of mastocytosis associated with either good or bad prognosis, respectively.[377] In 2001, the WHO classification of mastocytosis (updated in 2008) proposed the usage of one major (i.e. conventional histopathology) and four minor criteria (i.e. morphology, immunophenotype, molecular and biochemical criteria) for the diagnosis of SM; in addition, it defined seven major categories of mastocytosis, based on a set of "B"- and "C"-findings which reflect the variable clinical and biological features of mastocytosis, together with the heterogeneous treatment requirements associated with distinct variants of the disease.[7] More recently, growing knowledge about the pathophysiology of the disease exists which has led to the general belief that this is yet an evolving classification, because current WHO 2008 criteria may lead to frequent misdiagnoses, particularly among some infrequent subtypes of the disease, at the same time other highly prevalent disease categories (e.g. ISM) still remain heterogeneous, as regards both prognosis and treatment requirements.[337,339,356] Overall this points out the need for more refined diagnostic criteria and the definition of new variants of the disease, which show unique clinical (ISMs-), prognostic (e.g. ISMs+ with multilineage BM involvement by the *KIT* D816V mutation) and molecular (e.g. WDSM) features together with a distinct clinical behavior.[337,338]

Despite an increasing number of publications have focused on the analysis of the clinical, histopathological and biological features of the distinct variants of systemic mastocytosis, at present little is known about the molecular and biological mechanisms responsible for such heterogeneity, specially among those patients that carry the (same) D816V *KIT* mutation. In fact, most of these studies have either considered SM as a single entity, or they were focused on a single diagnostic subtype of mastocytosis.[113,305-307,320,321,378] Given the impact that genetic and microenvironmental alterations have on the gene and protein expression profiles of neoplastic cells, as well as their frequent contribution to the diagnosis, classification

and prognostic stratification of patients, and the understanding of the biological basis of neoplastic disorders,[379-383] in the present work we investigated the gene and protein expression profiles of BM mast cells from patients suffering from distinct subtypes of SM. Through this strategy, we aimed at gaining new insights on the biological mechanisms underlying the heterogeneity of SM and on determining their impact and potential utility in the diagnostic work-up and prognostic stratification of the disease.

In the following sections of the general discussion of this doctoral thesis, the potential significance of the most relevant results obtained during our work, is separately discussed for each of the proposed specific objectives.

4.1. Immunophenotypic characteristics of BM mast cells from distinct variants of SM.

The first goal of our work focused on a detailed analysis of the immunophenotypic profile of BM mast cells in the largest series of SM patients reported in the literature (n=123), at the time the study was done; based on this strategy we analyzed substantial numbers of patients which were representative of the distinct subtypes of the disease, in comparison to normal/reactive BM mast cells.

Overall, our results largely confirmed previous observations describing aberrant expression of CD2 and/or CD25 on BM mast cells from the great majority of the patients.[113,122] However, despite both proteins are considered as minor diagnostic criteria for SM, we were not able to detect expression of CD2 on BM mast cells from more than one fourth of the patients, whereas CD25 was found in virtually every case (>90%). It could be argued that such discrepancy could be due to the fact that in our study, expression of CD2 was assessed using a monoclonal antibody conjugated with fluorescein isothiocyanate (FITC), a fluorochrome which has been previously associated with a lower sensitivity than phycoerythrin (PE), particularly among cases where dim expression of CD2 is observed.[112] However, our results fully support more

recent observations which suggest that aberrant expression of CD25 is a more sensitive criterion for the diagnosis of SM than that of CD2.[322] In fact, in our cohort of patients only one case with (dim) expression of CD2 in the absence of CD25 was detected, this case corresponding to a WDSM patient who lacked any activating *KIT* mutation at codons 815-819; by contrast, more than a quarter of all SM patients who tested positive for CD25, showed no CD2 expression. Similarly to what had been previously described in smaller cohorts of patients,[115,124,320,321] a significantly increased expression of both activation (e.g. CD69) and degranulation (e.g. CD63 and CD203c) related molecules, as well as up-regulation of the CD59 complement regulator protein, was also found among our SM patients.

Interestingly, when the distinct subtypes of SM were separately analyzed, three clearly distinct immunophenotypic profiles were found, which were associated with specific subgroups of patients, namely: i) the most common good-prognosis categories of SM (ISMs+ and patients with clonal mast cell activating disorders – cMCAD – meeting the WHO criteria for ISM and therefore corresponding to ISMs-[338]); ii) WDSM, and; iii) SM cases with poor-prognostic subtypes of the disease (ASM and MCL).

Overall, the most recurrently detected immunophenotypic pattern corresponded to ISM patients (with or without skin lesions). These patients displayed most of the immunophenotypic features which have been classically associated with SM. This is probably due to the fact that these cases represent the most frequent forms of the disease,[325,327] and therefore a global analysis of SM patients would largely reflect the PEP of these particular variants of SM. In more detail, good-prognosis ISM patients typically showed a mature (e.g. FcεRI^hi) immunophenotype associated with aberrant expression of both CD2 and CD25, and overexpression of mast cell activation molecules like CD69 and the CD64 high-affinity IgG receptor (FcγRI), this later marker being absent in normal resting BM mast cells,[122] but expressed upon IFN-γ exposure.[148] Similarly, BM mast cells from patients with ISMs+ and ISMs- also showed increased expression of the HLA-DR and HLA-DQ major histocompatibility complex (MHC) class II

molecules, which are also absent in resting mouse and human mast cells, but up-regulated on activated mast cells isolated from tissues infected with pathogens and/or stimulated with cytokines (e.g. TNF-α and IFN-γ) and LPS.[213,214,230,384,385] In line with this activation-related PEP, up-regulation of molecules associated with mast cell degranulation (e.g. the lysosomal glycoprotein CD63 and CD203c) was also observed in this immunophenotypic subgroup of SM patients, probably reflecting an increased mediator release rate, which would account for the mast cell mediator release-associated symptoms typically displayed by these individuals.[338] Altogether, these results suggest that BM mast cells from patients with ISMs+ and ISMs- display similar features to those of mature activated mast cells, in addition to aberrant CD25 and CD2 expression.

Virtually every patient within these two subtypes of ISM (ISMs- and ISMs+) carried the D816V *KIT* mutation, and therefore potentially also constitutive activation of c-Kit.[257] Because of this, it could be speculated that the ectopic activated immunophenotype of BM mast cells from ISMs+ and ISMs- patients, which is typical of mast cells from peripheral tissues which have been naturally activated upon exposure to pathogens and other stimuli,[4,143] could probably be a consequence of the presence of the activating *KIT* mutation. This hypothesis is further supported by the absence of phenotypic differences between BM mast cells from ISMs- patients who displayed the D816V *KIT* mutation *vs.* other mutations in the tyrosine kinase loop domain of *KIT* (V815_I816ins), whereas the only ISMs- patient who showed a V560G mutation in the juxtamembrane domain of *KIT*, displayed clearly distinct phenotypic features (CD25$^{-/dim}$, cyBcl2^{hi}, CD2$^-$ and HLA-I^{dim}). However, the presence of the D816V *KIT* mutation *per se,* would not explain the distinct clinical features typically associated with the two subtypes of ISM,[338] suggesting that different activation-associated responses induced by the *KIT* D816V mutation would occur in BM mast cells from these two groups of ISM patients; such differences could potentially result from additional alterations and mechanisms. In line with this hypothesis, a significant direct correlation was observed

between serum baseline tryptase and CD64 expression (r^2=0.55, p=0.004) among ISMs-patients, but not within ISMs+ cases. In this regard, it should be noted that Woolhiser *et al.*[148] have shown that IgG molecules bound to surface CD64 (FcγRI) mediate mast cell degranulation, and that simultaneous FcεRI- and FcγRI-mediated MC activation leads to an additive degranulation at lower IgG and IgE concentrations. Altogether, these results suggest that increased expression of CD64 observed among ISMs-patients could result in a greater sensitivity and a more pronounced degranulation-associated phenotype, due to simultaneous FcεRI- and FcγRI-mediated mast cell activation; this could explain, at least in part, the severe systemic mast cell mediator release-associated symptoms (e.g. anaphylaxis) ISMs- patients typically display, despite their lower mast cell load.[337,338]

In contrast to ISMs- and ISMs+, BM mast cells from WDSM patients typically lacked expression of both CD2 and CD25, with only around one third of cases being either partially positive for CD25 or showing dim expression for CD2 in the absence of CD25. Of note, even in these later cases, the pattern of expression of CD2 and CD25 was significantly different from that typically detected in the other forms of the disease (CD2^{dim}/CD25$^-$ and CD2$^-$/CD25$^{-/+}$ *vs.* CD2-/CD25^{high} and CD2+/CD25^{high}); these results are in line with previous observations in both individual cases[265] and in small series of WDSM patients.[346] Furthermore, BM mast cells from WDSM patients also showed normal expression of the CD59 complement regulatory protein and of the CD63, CD69 and CD203c activation/degranulation-related molecules, which are typically described to be overexpressed on BM mast cells from SM patients.[123,124,320,321] In fact, BM mast cells from WDSM patients displayed an overall phenotype similar to that observed for mature resting normal BM mast cells (strong expression for both CD117 and FcεRI);[109,123] and aberrant mast cell phenotypes from WDSM patients were restricted to a few (overexpressed) intracellular antigens, such as cytoplasmic Bcl2, CPA and total tryptase. Increased expression of the anti-apoptotic Bcl2 protein suggests that an altered regulation of apoptosis could contribute to the pathogenesis of WDSM,

similarly to what has been reported for CM mast cells.[386,387] However, the most striking feature of WDSM BM mast cells was their increased cytoplasmic contents in mast cell enzymes (e.g. tryptase and CPA) which probably contributes to the typical hypergranulated morphological appearance[325] of these cells, and their abnormally increased size and internal complexity (FSC^{high} and SSC^{high} mast cells). Interestingly, increased expression of cytoplasmic total tryptase (pro and mature α/β-tryptase identified by the B12 antibody) was associated with unexpectedly low serum total tryptase levels, suggesting that spontaneous release of protryptase could be affected in WDSM patients. This is particularly true, if we consider that protryptase is spontaneously secreted by mast cells, and therefore reflects the mast cell burden, whereas mature tryptase is stored in cytoplasmic granules and it is only released in response to mast cell stimulation.[204] In line with this hypothesis, WDSM patients showed extremely low serum tryptase per BM mast cell (serum tryptase levels/%BM mast cell ratio), further supporting a decreased rate of release of tryptase (and potentially also other enzymes like CPA) in these patients. Of note, WDSM showed high levels of cytoplasmic (Cy) total tryptase (B12) in association with a normal total/mature tryptase (assessed by the G5 antibody) ratio (CyB12/CyG5 ratio), which suggests that the lower rate of release of protryptase could be due to an increased rate of intracellular protein processing, leading to a relative increase in the levels of the mature forms of the protein, which are typically released only upon mast cell stimulation;[204] if this holds true, an increased cytoplasmic expression of mature tryptase would occur. Alternatively, deregulation of the mechanisms controlling mast cell secretion/release could also occur in these patients. In line with this later hypothesis, a significantly increased expression of CD22 was observed in all subtypes of SM, including WDSM patients. This molecule belongs to the family of Siglec proteins, whose members (e.g. Siglec 8) have been previously described to be involved in the inhibition of histamine and prostaglandin D2 secretion and of Ca^{2+} influx, in mast cells.[388,389] Further studies, in which the expression of other cell surface inhibitory

molecules (e.g. CD200R, LAIR1, CD84 or CD300a)[188] is investigated, together with the evaluation of IgE-induced degranulation of BM mast cells from WDSM patients, are necessary to determine the exact mechanisms responsible for the increased cytoplasmic expression of mast cell enzymes in this subgroup of SM.

On top of all the above, it should be noted that clonal BM mast cells from most WDSM patients did not show *KIT* mutations, and that those few WDSM patients who carried *KIT* mutations (including a D816V+ case) displayed similar immunophenotypic profiles to those of their non-mutated counterpart; these findings suggest that BM mast cells from WDSM may carry additional mutations/genetic lesions involving other proteins downstream of c-Kit, which could specifically lead to the impaired secretory phenotype.

In contrast to other subgroups of SM, the poor-prognosis subvariants of the disease (ASM and MCL), typically displayed bright expression of CD25, frequently in the absence of CD2. This aberrant phenotype was associated with increased expression of the CD123 and HLA-DR antigens, which are typically expressed by immature BM mast cells;[22,96,123] in line with this, BM mast cells from ASM and MCL patients also showed lower expression of CD117, HLA-I and FcεRI, together with abnormally low levels of cytoplasmic tryptase and CPA, in association with decreased light scatter (SSC and FSC) features. Such immunophenotypic profile has been described as typical of immature mast cells.[17,96,123] Overall, these results suggest that BM mast cells from poor-prognosis variants of SM (ASM and MCL) display a relatively immature phenotype. Interestingly, the phenotypic differences observed between these poor-prognosis categories of SM and ISM, could not be explained on the basis of a different *KIT* mutational pattern, since most ASM and MCL patients displayed the same D816V *KIT* mutation as ISM cases. However, in contrast to ISM, MCL and ASM typically carried the D816V *KIT* mutation in almost all BM myeloid cell populations, including CD34+ HPC,[257] whereas among ISMs- and ISMs+ patients the *KIT* mutation was typically restricted to BM mast cells. These results suggest that occurrence of an extended clonal hematopoiesis in SM

with multilineage involvement, would be associated with an earlier maturation blockade, among the more aggressive forms of SM. In line with this hypothesis, when we compared the good- (ISM) *vs.* poor-prognosis categories of SM (ASM and MCL), the later typically showed a lower frequency of skin lesions with a greater tumor load in the BM and other lymphoid tissues,[359] further supporting the existence of an underlying maturation blockade at relatively early stages, prior to the migration of recently produced mast cells to the skin.[36] Additionally, these observations also suggest that an increased chemotaxis towards the spleen and/or the liver (typically involved in aggressive forms of the disease) and a decreased homing to the skin (more frequent in indolent SM and WDSM), could be a trait of the initial steps of mast cell differentiation. Further molecular/genetic studies are necessary to elucidate whether such maturational blockage and altered pattern of homing of clonal mast cells could result from the co-existence of additional genetic lesions involving a D816V[+] hematopoietic progenitor cell in patients with poor-prognosis SM and/or to the presence of different patterns of expression of mast cell-associated adhesion molecules and chemokine receptors.

Regarding SM-AHNMD cases, ISM-AHNMD and ASM-AHNMD patients displayed similar phenotypic profiles for the great majority of the proteins analyzed, to those exhibited by their non-AHNMD counterparts, in line with what could be expected.[7]

Based on the overall different PEP found among the distinct diagnostic variants of SM, for expression of those phenotypic markers analyzed, a scoring algorithm was built which predicted for the different subtypes of SM (WDSM *vs.* ISMs-/ISMs+ *vs.* ASM/MCL) with high sensitivity and specificity; this finding supports the potential role of MFC immunophenotyping of BM mast cells from SM patients, not only for the diagnosis, but also for the classification of the disease.

In summary, our results confirm that BM mast cells from SM are phenotypically heterogeneous, with three clearly distinct maturation-associated profiles; such protein expression profiles of BM mast cells are related to both molecular and prognostic

subtypes of mastocytosis. Accordingly, more immature immunophenotypic patterns are typically found in ASM and MCL, while the nature of mature BM mast cell phenotypes associated with good-prognosis subtypes of SM - activated (ISMs-/ISMs+) *vs.* resting (WDSM), BM mast cell phenotypes - appear to depend on the presence *vs.* absence of the D816V *KIT* mutation.

4.2. Association between the immunophenotypic profile of BM mast cells in SM and the degree of involvement of hematopoiesis by the *KIT* D816V mutation.

As already referred above, aggressive forms of mastocytosis (e.g. ASM and MCL), systematically carry the D816V *KIT* mutation in the different myeloid or both myeloid plus lymphoid compartments of BM hematopoietic cells, other than mast cells;[257] in addition, these patients typically display in common, an immature immunophenotypic profile. Conversely, ISM patients (with or without skin lesions) more frequently exhibit the *KIT* D816V mutation restricted to the pathological BM mast cell population,[257] and they show a mature activated BM mast cell immunophenotypic profile. However, several nuances were observed in this particular pattern, as ISMs+ patients who displayed hepatomegaly and/or splenomegaly exhibited slightly more immature immunophenotypic features (e.g. lower expression of the FcεRI IgE high-affinity receptor), together with higher serum tryptase levels, both features being more frequently detected among aggressive forms of the disease (e.g. ASM). In line with these findings, recent studies have shown that in around 20% of ISMs+ cases, the *KIT* mutation is also detected in myeloid and lymphoid BM cell compartments other than BM mast cell, similarly to what occurs in ASM patients;[257] most importantly, this extended involvement of hematopoiesis by the *KIT* D816V mutation has been shown to be the most powerful independent prognostic factor for progression of ISM to more aggressive forms of the disease (e.g. ASM, MDS or AML).[356] Altogether, these observations raised the question about whether there would be an association

between the immunophenotypic features of BM mast cells from ISMs+ patients and the underlying degree of BM involvement by the D816V *KIT* mutation.

In order to gain insight into this potential association, we performed an unsupervised analysis of the PEP of BM mast cells in three different series of patients and/or groups of molecules. Interestingly, in all three sets of analyses, a similar hierarchical organization of the cases was observed, with two major groups of cases: ISM with *KIT* mutation restricted to the BM mast cell compartment (ISMs- patients and most ISMs+ patients) and cases with extensive involvement of the hematopoiesis by this mutation (ASM and some ISMs+ patients); of note, such classification of SM patients, rather than reflecting distinct clinical features of the disease *per se*, indicates that patients with multilineage *KIT* mutation would share unique immunophenotypic features, regardless of the diagnostic subtype of the disease. A more detailed analysis of the pattern of expression of individual molecules within each of the two groups of patients, revealed that those ISMs+ cases with *KIT* mutation restricted to the BM mast cell compartment exhibited a mature (FcεRI[hi]) activated (e.g. strong CD203c expression)[96,104] PEP similar to that of ISMs- patients. Conversely, ISMs+ patients who displayed multilineage involvement shared several features with ASM patients, who typically show also myeloid or myeloid plus lymphoid BM involvement by the *KIT* mutation.[257] Similarly to ASM, in these later ISMs+ cases, an increasing number of BM cell populations bearing the mutation was associated with a higher BM mast cell burden and increased serum baseline total tryptase (sBt) levels. In addition, ISMs+ cases carrying multilineage *KIT* mutation also displayed decreased expression of proteins known to be acquired during BM mast cell maturation (e.g. FcεRI, CD45 and mast cell enzymes CPA and total tryptase),[16-18,20,106,107] in association with lower light scatter properties,[103] features that are compatible with a more immature mast cell phenotype.

Furthermore, decreased expression of the CD9 tetraspanin molecule was also observed among ISMs+ patients carrying multilineage *KIT* mutation, who are at higher

risk of progression to more aggressive disease (e.g. ASM, MDS and AML). Interestingly, downregulation of CD9 has been described to correlate with tumor progression in several types of cancer[390] due to the fact that it has the ability to inhibit several metastasis-promoting processes, including integrin-mediated motility; therefore, down-regulation of this molecule could be associated with an increased invasion capacity.[390] This is particularly interesting when considering that the more aggressive forms of SM (e.g. ASM) typically display massive mast cell infiltration in organs/tissues in which mast cells are poorly represented under physiological conditions (e.g. spleen and liver),[313] typically leading to splenomegaly and/or hepatomegaly and, in the most extreme cases, to the functional impairment of these organs.[359] However, further studies are required to necessary to determine the role of the expression of CD9, and other tetraspanins (e.g. CD81), as well as other adhesion/migration-associated molecules, in the distinct pattern of organ/tissue involvement in SM.

Overall, the results discussed above suggest that multilineage involvement by the *KIT* mutation in ISM patients, may be associated with an early blockade of mast cell maturation, leading to more immature BM mast cell features and a less activated phenotype. Interestingly, as all ISMs+ patients carried a common *KIT* mutation, this genetic lesion alone could not fully account for the phenotypic differences observed within ISM patients. In turn, it could potentially result from coexisting genetic and/or epigenetic events other than the D816V+ mutation occurring in *KIT* mutated HPC, similarly to what has been previously suggested for polycythemia vera, essential thrombocythemia and primary myelofibrosis patients who share an identical activating somatic mutation of the *JAK2* tyrosine kinase gene ($JAK2^{V617F}$), but display distinct clinico-biological features.[391,392] Alternatively, constitutive activation of c-Kit due to the presence of the D816V *KIT* mutation, could lead to variable biological outcomes depending on the maturational stage of the cell in which the mutational event occurs - either a HPC or a more mature mast cell precursor -, as a result of the distinct transcriptional machinery which is active at the different stages of differentiation of

mast cells.[76] In line with this latter hypothesis, expression of MITF-A, an alternative splicing isoform of MITF (a transcriptional factor which plays an important role in the final stages of mast cell differentiation in e.g. regulating the transcription of tryptase gene in human mast cells[393]), has been recently reported to be regulated by c-Kit signaling.[394] Despite all the above, and the variable PEP of clonal mast cells in ISM patients with or without multilineage involvement of BM cells by the *KIT* mutation, further studies are necessary to determine the precise mechanisms leading to the accumulation of mast cells in the BM with an earlier maturation blockade.

As previously mentioned, despite the great majority of ISMs+ patients show an overall survival which is similar to the remaining non-SM population,[327] a subset of these patients who have "indolent" variants of the disease according to the WHO 2008 criteria, show progression to more aggressive disease (e.g. ASM, MDS or AML).[356] Among those parameters which contribute to identify patients at risk of progression, the presence of multilineage *KIT* mutation has been shown to be the most powerful independent prognostic factor.[356] However, determination of the degree of involvement of BM hematopoiesis by the *KIT* mutation currently requires complex molecular analyses of highly-purified cell populations;[257] such approaches are not easily and widely available in most routine diagnostic laboratories, and they can only be performed in highly specialized reference centers. Therefore, the development of strategies and algorithms that would allow for the identification of those ISM patients at risk of progression (e.g. ISM cases that carry multilineage involvement of hematopoiesis by the *KIT* mutation), is mostly welcome. Since BM mast cells from ISMs+ patients with multilineage BM involvement show distinct (e.g. more immature) immunophenotypic features than other ISM patients, we wondered whether this association between the immunophenotype of BM mast cells and the degree of clonal hematopoiesis could be used as a tool for the diagnostic screening of multilineage involvement of hematopoiesis by the *KIT* mutation in SM.

In order to accomplish this goal, we developed a score-based class prediction model, based on those immunophenotypic markers which were tested in a significant number of patients (>80%), that showed a high discrimination power, as assessed by receiver operating characteristic (ROC) curves. This score was first tested in a training set of ISMs+ patients, in whom such phenotypic variables were simultaneously analyzed. Based on the proposed model, multilineage *KIT* mutation was identified on the basis of an increased BM infiltration by clonal mast cells with aberrant expression of CD25 in association with a FcεRI^{lo}, FSC^{lo}, SSC^{lo} and CD45^{lo} immature immunophenotype, in the absence of a coexisting population of BM mast cells with a normal phenotype. In addition, the classifier was validated using two other, distinct subsets of patients whose BM mast cells had been analyzed with two different flow cytometer instruments (either a 4-colour FACSCalibur or a 8-colour FACSCanto II, Becton/Dickinson Biosciences, San Jose, CA, USA) and stained with distinct monoclonal antibody (mAb) clones and/or fluorochrome conjugates. Once again, the scoring system proved to be highly efficient for the classification of ISM patients according to the degree of involvement of BM hematopoiesis by the *KIT* mutation, in both the training and validation sets of patients. Furthermore, the proposed algorithm was not only able to correctly classify ISMs+ cases, but also other SM patients, into cases with mast cell-restricted *vs.* multilineage BM involvement by the *KIT* D816V mutation, regardless of the diagnostic subtype of the disease. These results suggest that the immunophenotypic features of mast cells could be used as a surrogate marker for the identification of SM patients carrying multilineage *KIT* mutation, without previous knowledge about the diagnostic subtype of the disease. Of note, this approach can be easily implemented in routine diagnostic flow cytometry laboratories.

Further validation of the classification algorithm proposed was performed, through determination of its impact on progression-free survival (PFS) of ISMs+ patients. As could be expected, the new immunophenotypic algorithm here proposed had a similar (significant) impact on PFS of ISMs+ patients, to that of conventional molecular

assessment of multilineage *KIT* mutation. Nevertheless, a prolonged follow-up of these patients is necessary to determine which of the two methods has a higher prognostic impact among ISMs+ patients, as none of the discrepant cases progressed at the study closing date, due to the smaller follow-up period of this (small) group of patients, compared to that of those cases that progressed. Altogether, these findings support the utility of immunophenotyping of BMMC as a surrogate marker to predict for the degree of clonal involvement of hematopoiesis in the BM of SM patients, independently of the clinical subtype the disease, at the same time they point out the need to perform a careful follow-up of those patients with immature BMMC PEP, specially among those patients with an expected long-term disease.

Despite the association between the degree of involvement of BM hematopoiesis by the *KIT* D816V mutation and the prognosis of ISMs+ patients is currently well established, the biological mechanisms which determine disease progression among these patients, and that are ultimately responsible for their variable outcome, remain largely unknown.[356] As evolution to more aggressive disease is a slow process, it could be hypothesized that an increasing BM involvement by the *KIT* mutation of e.g. CD34+ HPC (and therefore of the overall mutated cell load) could be observed throughout time, as a result of a proliferative/survival advantage conferred by this mutation; thus mutated cells would slowly replace normal hematopoiesis, with an increasing probability of occurrence of secondary/additional genetic and epigenetic changes in the mutated BM HPC, ultimately leading to the development of more aggressive disease like ASM, MDS or AML, in a similar way to what has been described during the evolution of MDS to AML.[395,396] Moreover, clonal BM cells (either mast cells or CD34+ HPC) could progressively compete and replace their normal counterpart in the BM stromal cell niches, ultimately leading also to more aggressive disease, as previously suggested for patients with monoclonal gammopathy of undetermined significance (MGUS) who progress to multiple myeloma.[397] Alternatively, the *KIT* mutation could target a different stem/precursor cell in patients who carry multilineage mutation,

leading to an increased predisposition to acquire distinct additional genetic events that favor subsequent progression to more aggressive forms of the disease.[356]

In summary, our results show that the immunophenotypic profile of BM mast cells from patients with SM closely reflects the degree of BM involvement by the *KIT* D816V mutation, as more immature BM mast cell features are typically found in ISMs+ patients who carry multilineage clonal hematopoiesis *vs.* ISMs- and ISMs+ cases who show the same *KIT* mutation, but restricted to the BM mast cells. Furthermore, these distinct immunophenotypic profiles of BM mast cells can be used as a surrogate marker for the diagnostic screening of multilineage *KIT* mutation among ISMs+ patients, therefore contributing to the prognostic stratification of this group of ISM patients.

4.3. The GEP of (highly-purified) BM mast cells from distinct variants of SM carrying the D816V *KIT* mutation.

Despite SM comprises a heterogeneous group of well-defined entities with unique clinical and histopathological features and therapeutic requirements,[325,338,356] little is known regarding the molecular mechanisms responsible for such heterogeneity. In fact, except for WDSM patients, the great majority (>90%) of SM patients carry a common genetic lesion, the *KIT* D816V activating mutation.[257] In line with this, several groups have recently reported that the presence of multilineage involvement of BM hematopoiesis[257,356] and the occurrence of mutations in gens other than *KIT* (e.g. *TET2* and *NRAS*),[278,281] could be linked to more aggressive forms of systemic disease (e.g. ASM, MCL and ISMs+ patients at risk of progression). Although these findings have contributed to gain insight into the potential molecular mechanisms underlying the heterogeneity of SM, the specific biological functions and pathways associated with each of the distinct variants of the disease, remain largely elusive. In order to address this issue, we applied cDNA-oligonucleotide arrays to the analysis of the gene

expression profiles of highly-purified mast cells isolated from BM samples of SM patients with distinct variants of the disease who had in common the D816V *KIT* mutation, in parallel to BM mast cells from a group of control subjects, including both normal and reactive samples. To the best of our knowledge this is the first time the GEP of purified BM mast cells from SM patients is analyzed.

Since c-Kit mediated signaling has been widely described to be influenced by the cell's microenvironment (e.g. cytokines),[23,36,76] inclusion of reactive hyperplastic samples as control samples, would lead to the exclusion of those genes which could be deregulated as a consequence of changes in the BM microenvironment and that do not result from deregulated GEP linked to the genetic lesions underlying mastocytosis. Furthermore, the usage of isolated mast cells allowed us to overcome serious handicaps underlying previous reports about the GEP of mastocytosis patients, which used either mononuclear BM cells[305] or whole peripheral blood samples[306,307], and whose results were therefore highly influenced by the variable cellular composition and the heterogeneous mast cell load of individual samples. In line with this, D'Ambrosio *et al.*[305] found a significant correlation between the gene expression levels of α-tryptase, using BM mononuclear cells, and serum baseline total tryptase, a known indicator of mast cell burden.[203]

Overall, supervised analysis of the GEP of highly-purified BM mast cells from our patients showed distinct patterns for each of the three major diagnostic subtypes of SM investigated: ISMs-, ISMs+ and ASM, with a total of 915 deregulated genes that included a common imprint of 286 altered genes.

The signaling pathways which were activated by (SCF-induced) c-Kit activation play a key role in both the differentiation and the activation of MC; in fact, activation of c-Kit leads to the engagement of multiple downstream signaling pathways involved in mast cell growth, differentiation, survival, chemotaxis and cytokine production.[16,49] Since the presence of the *KIT* D816V mutation was a common trait to all patients included in this part of the study, the presence of an activating *KIT* D816V mutation

would potentially be reflected by a common GEP. Accordingly, the common gene signature to all variants of SM analyzed was characterized by a general up-regulation of genes involved in cytokine-mediated signal transduction, including target genes from the JAK/STAT pathway, like the *BCL2L1* anti-apoptotic gene which codes for the Bcl-xL protein, previously described to be strongly expressed in BM mast cells (but not in mast cells from skin lesions) of SM patients.[386,398] Surprisingly, a significant enrichment was found in this common gene expression signature, for type I IFN-dependent response genes. This is particularly remarkable given the fact that patients included in the study were not treated with interferon. Interestingly, most of these IFN-induced genes (e.g. *OAS1, OAS2, MX1, MX2* or *CCL3*) were also previously described to be up-regulated upon MC infection by viruses,[399,400] suggesting that c-Kit downstream signaling could play an important role in mast cell responses to viral infections. In line with this hypothesis, previous studies reported that infection of endothelial cells by the Kaposi's sarcoma herpesvirus (KSHV) induces up-regulation of c-Kit, and that the inhibition of either c-Kit activation or its downstream effectors, reverses the characteristic KSHV-induced morphological transformation typically displayed by the infected cells.[401,402] Furthermore, an overall enrichment in genes involved in both innate and inflammatory immune responses was also detected in common to all three subtypes of SM. Among other genes, this included increased expression of the complement regulatory proteins *CLU* (clusterin) – also previously reported to be up-regulated on SM BM mononuclear cells-,[305] *CFH* (complement factor H) and *SERPING1* (plasma protease C1 inhibitor), in all subtypes of SM. These findings are in line with previous studies about the PEP of SM patients which reported increased expression of membrane-bound complement inhibitory proteins like CD35 (complement receptor 1, CR1) and CD59 (membrane inhibitor of reactive lysis, MIRL),[124] and they could reflect a self-defense mechanism of mast cells from complement-dependent cytotoxicity, similar to what has been described for other types of tumors [403,404].

In addition to the common GEP, deregulation of different biological processes was specifically associated with each of the three diagnostic subtypes of the disease investigated. Even though ISMs- and ISMs+ BM mast cells shared a similar GEP as regards some biological functions (e.g. T-cell costimulation), ISMs- patients displayed a higher number of specifically deregulated genes, including the *CD2* adhesion molecule. Functional enrichment analysis of ISMs- BM mast cell GEP also revealed significant up-regulation of transcripts involved in lipid metabolism. Interestingly, activation of mast cells is widely known to induce an increased phospholipid metabolism associated with a greater production of arachidonic acid metabolites (e.g. prostaglandin D2 and leukotriene C4),[147,179] which are partly responsible for the mast cell-mediator release-associated symptoms that are typically displayed by ISMs- patients.[338,405,406]

Despite the overall number of deregulated genes in ISMs- and ISMs+ was similar, most genes found to be deregulated in ISMs+ patients were also altered in other subtypes of the disease (e.g. ASM). This could be due to the inclusion within this latter group of patients of cases with a variable degree of hematopoietic involvement by the *KIT* D816V mutation with both multilineage and mast cell-restricted *KIT* mutated patients. As discussed above, the extent of involvement of hematopoiesis by the *KIT* mutation has a significant impact on the immunophenotypic features of BM mast cells, cases with the mutation restricted to the mast cell compartment being more similar to ISMs-, while patients with multilineage involvement exhibit an immunophenotype comparable to that of ASM. Despite this heterogeneity, ISMs+ BM mast cells specifically displayed an increased expression of genes involved in gene transcription and protein processing, probably reflecting an increased rate of *de novo* production of mast cell mediators such as the CCL2 and CCL3 pro-inflammatory chemokines.[49,143] Overall, the distinct GEP displayed by indolent forms of the disease suggests that, despite both ISMs- and ISMs+ BMMC carry the same *KIT* D816V mutation and show an overall activated mast cell immunophenotypic profile, the type of response to this constitutive activation, mediated by c-Kit, is different: increased lipid metabolism *vs.*

protein production; such differences could probably result from the concurrence of distinct mechanisms that modulate mast cell activation via either Kit, FcεRI and/or HLA-II signaling, or because of a distinct genetic background in both groups of ISM patients. Altogether, these results further support previous reports which indicate that these two groups of ISM patients could represent distinct variants of ISM, and should therefore be classified as separate entities.[338,407]

Activation of c-Kit is known to also regulate adhesion of mast cells to the connective tissue matrix.[26,131] In this regard, significant deregulation of the expression of cell adhesion-related genes was observed in both ISMs+ and ASM. Nonetheless, a distinct expression pattern was observed between aggressive and indolent forms of the disease: ASM BM mast cells displayed a higher frequency of down-regulated transcripts, at the same time they showed normal expression of genes up-regulated in ISMs+ patients (e.g. *COL12A1* - collagen, type XII, alpha 1 - and *CDH12* - cadherin 12, type 2-). Since one of the major features of ASM is the frequent involvement of organs and tissues other than BM and the skin (e.g. spleen, liver, lymph nodes and/or peripheral blood), whereas presence of cutaneous lesions is a hallmark of ISMs+ patients,[7,8] such distinct pattern of expression of adhesion-related genes could potentially contribute to explain, at least in part, the different patterns of organ/tissue involvement observed in both subtypes of SM.

Overall, BM mast cells from D816V *KIT*-mutation[+] ASM patients also showed a unique GEP, which was clearly different from both ISMs- and ISMs+ cases carrying the same *KIT* mutation. In general, our results point out the potential deregulation of two distinct but interconnected mechanisms in ASM BM mast cells: i) inhibition of apoptosis, as a result of the up-regulation of anti-apoptotic-associated genes like *BFAR* (bifunctional apoptosis regulator), and; ii) significant deregulation of cell cycle, including increased expression of genes involved in cell-cycle checkpoints like cyclin B1 (*CCNB1*), often found to be overexpressed in various human tumors, including lung cancer,[408] astrocytomas[409] and esophageal squamous cell carcinoma,[410,411] in

association with an increased cell proliferation.[412] In fact, a slightly increased mast cell proliferation was observed for all variants of SM analyzed, ASM patients being the only cases who exhibited significantly increased percentages of S+G_2/M phase cells. Altogether these findings suggest that the pathogenesis of aggressive forms of SM could rely on different mechanisms, associated with increased proliferation and/or inhibition of apoptosis. Further studies, in larger cohorts of ASM patients are required to confirm the existence of distinct mast cell GEP within ASM patients, and their potential association with specific genetic lesions other than the *KIT* D816V mutation, distinct clinical forms of the disease and unique patterns of response to therapy.

Despite all the differences here reported in the GEP of ISMs- and ISMs+ BMMC, as well as the clinical heterogeneity associated with ASM patients, a signature of 22 genes allowed for the classification of indolent *vs.* aggressive forms of the disease with a high overall accuracy. Since this signature also allowed for correct identification of ISMs+ cases carrying multilineage BM involvement by the *KIT* D816V mutation, and progression of these later cases to ASM is not a rare event,[356] such gene signature could potentially serve in the future as prior knowledge for the discovery of candidate biomarkers for adequate monitoring of those patients at higher risk of progression (e.g. quantification of serum CCL23, IL1β or IL13 levels). In this regard, previous studies have reported an association between the -1112C/T polymorphism of the *IL-13* promoter gene, and both "hypertranscription" of this cytokine and systemic forms of mastocytosis.[283] Further studies, in larger cohorts of patients are necessary to determine the potential utility of such prognostic markers.

A major concern regards the functional impact of GEP studies on protein expression. In order to validate the functional impact of GEP of BM mast cells, we assessed in parallel the mRNA and protein expression levels (using MFC immunophenotypic studies) of a subgroup of 10 genes (*CD2, CD4, CD33, CD52, FCER1A, HLA-DRB1, HLA-DRB5, IL2RA, IL3RA* and *LAIR1)* which code for proteins that are primary localized on the cell surface membrane and that have not been described to

be stored in BM mast cell granules. Such validation studies were performed in an enlarged group of patients. Overall, our results showed a significant positive correlation between mRNA and protein levels for all genes tested, except for the *FCER1A* gene, for which an inverse correlation was observed; these latter results are consistent with previous reports on the metabolic rate of FcεRI on basophils, which suggest that production of FcεRI is modulated during cell maturation and that once the cell is mature and exhibits high expression of membrane FcεRI, the rate of synthesis of the receptor slows down.[413] In addition, expression of FcεRI on the cell surface is not only the result of the expression of FcεRIα, but it also depends on the rate of synthesis of FcεRIβ and FcεRIγ, as well as on the levels of free IgE.[413]

In summary, in the present study we show for the first time that the presence of the activating *KIT* D816V mutation in BM mast cells from distinct subvariants of SM confers a common GEP to these cells; such common GEP is mainly characterized by up-regulation of genes involved in innate and inflammatory immune responses, including several IFN-induced genes involved in anti-viral responses. In addition, a distinct and unique GEP was also observed for each of the three subtypes of SM analyzed, such profile reflecting an increased lipid *vs.* protein metabolism in ISMs- *vs.* ISMs+ cases, together with inhibition of apoptosis and/or an altered cell cycle regulation in ASM patients, highlighting the potential nature of relevant secondary genetic lesions and microenvironmental alterations specific for these subgroups of SM patients.

Chapter 5 |

CONCLUDING REMARKS

The knowledge accumulated during the past few years about mast cell physiology and its alteration in mastocytosis, has dramatically changed the diagnosis and treatment of systemic mastocytosis patients, at the same time it has lead to the definition of distinct variants of the disease with different clinical and prognostic features, along with diverse therapeutic requirements. However, the basis for such disease heterogeneity remains largely unknown. In order to gain insight into the biological mechanisms which contribute to the variable clinical behavior of SM patients, we aimed at a detailed analysis of both the protein and gene expression profiles of BM mast cells from patients suffering from distinct variants of the disease, particularly focusing on those patients that carry the *KIT* D816V mutation. Based on the work done during this doctoral thesis, the following conclusions can be drawn:

- Regarding the immunophenotypic characteristics of BM mast cells from distinct variants of SM.

1. BM mast cells from SM are phenotypically aberrant but heterogeneous, with three clearly distinct maturation-associated profiles. Such profiles reflect distinct molecular and prognostic subtypes of mastocytosis: more immature immunophenotypic patterns are typically found in ASM and MCL, while mature activated and resting BM mast cell features are associated with good-prognosis subtypes of SM, depending on the presence or absence of the D816V KIT mutation, respectively.

- With respect to the potential relationship between the immunophenotypic profile of BM mast cells and the degree of involvement of hematopoiesis by the KIT D816V mutation.

2. BM mast cells from SM patients, including ISM, show an immunophenotypic profile which reflects the degree of involvement of BM hematopoiesis by the

KIT D816V mutation. Accordingly, more immature BM mast cell features are typically found among ISMs+, ASM and MCL patients who have multilineage clonal hematopoiesis, whereas a mature activated immunophenotype is characteristically detected in ISM patients carrying the mutation restricted to the BM mast cell compartment.

3. Among ISM patients, coexistence of a high degree of BM infiltration by clonal mast cells displaying aberrant expression of CD25 together with an immature (FcεRI^{lo}, FSC^{lo}, SSC^{lo} and CD45^{lo}) immunophenotype, in the absence of normal BM mast cells, reflects the presence of an underlying clonal hematopoiesis. The combination of these parameters provides a highly efficient surrogate marker for the diagnostic screening of multilineage *KIT* mutation among ISMs+ patients, and consequently, it also potentially contributes to the prognostic stratification of this group of ISM patients.

- Regarding the GEP of (highly-purified) BM mast cells from distinct variants of SM carrying the D816V KIT mutation.

4. The presence of the activating D816V *KIT* mutation in BM mast cells from distinct subvariants of SM is associated with a common GEP, distinct from that of normal/reactive mast cells. Such GEP is mainly characterized by up-regulation of genes involved in innate and inflammatory immune responses, including IFN-induced genes involved in anti-viral responses.

5. D816V+ BM mast cells from ISMs-, ISMs+ and ASM patients show distinct and unique GEP, such specific GEP reflecting an increased lipid *vs.* protein metabolism in ISMs- *vs.* ISMs+ cases, together with the inhibition of apoptosis and/or an altered cell cycle regulation among ASM patients. Altogether, these results point out the potential nature of relevant secondary genetic and epigenetic lesions and/or

microenvironmental alterations, shared by patients included in these three different diagnostic subgroups of SM.

Chapter 6 |

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