



Immunoprofiling Reveals Novel Mast Cell Receptors and the Continuous Nature of Human Lung Mast Cell Heterogeneity

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Background: Immunohistochemical analysis of granule-associated proteases has revealed that human lung mast cells constitute a heterogeneous population of cells, with distinct subpopulations identified. However, a systematic and comprehensive analysis of cell-surface markers to study human lung mast cell heterogeneity has yet to be performed.

Methods: Human lung mast cells were obtained from lung lobectomies, and the expression of 332 cell-surface markers was analyzed using flow cytometry and the LEGENDScreen™ kit. Markers that exhibited high variance were selected for additional analyses to reveal whether they were correlated and whether discrete mast cell subpopulations were discernable.

Results: We identified the expression of 102 surface markers on human lung mast cells, 23 previously not described on mast cells, of which several showed high continuous variation in their expression. Six of these markers were correlated: SUSD2, CD49a, CD326, CD34, CD66 and HLA-DR. The expression of these markers was also correlated with the size and granularity of mast cells. However, no marker produced an expression profile consistent with a bi- or multimodal distribution.

Conclusions: LEGENDScreen analysis identified more than 100 cell-surface markers on mast cells, including 23 that, to the best of our knowledge, have not been previously described on human mast cells. The comprehensive expression profiling of the 332 surface markers did not identify distinct mast cell subpopulations. Instead, we demonstrate the continuous nature of human lung mast cell heterogeneity.

Keywords: human lung mast cells, heterogeneity, chymase (CMA1), carboxypeptidase A3 (CPA3), SUSD2, CD38, FcεRI

INTRODUCTION

Heterogeneity among mast cells has been known for a long time and was first attributed to differential expression of proteoglycans in rodent mast cells, which gave them distinct staining patterns (1). This led to the division of rodent mast cells into connective tissue mast cells and mucosal mast cells. In humans, mast cell heterogeneity has been based on the expression of mast cell proteases, i.e., cells expressing tryptase only (MC_T) and those expressing both tryptase and chymase (MC_{TC}) as well as carboxypeptidase A (2, 3). These subtypes have been defined using immunohistochemistry, a method that produced binary results, that is, the absence or presence of expression. The MC_{TC} subtype is more predominant in connective tissues such as the skin, while the MC_T subset is more prevalent in mucosal surfaces such as the airways and gastrointestinal tract (4).

Mast cells are found in the human lungs in all different compartments, i.e., under the epithelium, in smooth muscle bundles, around pulmonary vessels, in the parenchyma and in close proximity to sensory nerves (5). Human lung mast cells (HLMCs) have several important functions in health and diseases, such as host defense, induction of acute inflammatory responses, vascular regulation, bronchoconstriction and tissue remodeling (6–9). The heterogeneity of HLMCs was first suggested to be related to differences in their size and function (10), where a heterogeneity in the response to secretagogues also was reported (11). It was described that the protease expression within HLMCs differ, with the MC_T being the predominant subtype except around pulmonary vessels, where the MC_T and MC_{TC} subtypes are found in equal numbers (2). However, the heterogeneity among HLMCs goes beyond size and protease expression, as demonstrated by the differential expression of certain mast cell-related markers (FcεRI, IL-9R, 5-LO, LTC₄S, etc.) among the MC_T and MC_{TC} populations in different lung compartments (12).

Mast cell heterogeneity has primarily been studied in a binary manner using immunohistochemistry, describing the absence or presence of a given marker. Here, we used a quantitative flow cytometry-based approach to study HLMC heterogeneity, profiling the expression of 332 surface markers and intracellular staining of the proteases tryptase, chymase and CPA3. None of these markers distinctly divided the HLMCs studied into subpopulations. However, several markers showed a high degree of variation within the mast cell population with a nonclustered gradient expression pattern. Six of these markers correlated with each other, revealing the continuous nature of HLMC heterogeneity rather than separation into distinct subpopulations.

MATERIALS AND METHODS

Ethical Approval

The local ethics committee approved the collection of lung tissue from patients undergoing lobectomy, and all patients provided

informed consent (Regionala Etikprövningsnämnden Stockholm, 2010/181-31/2).

Cell Preparation

Single-cell suspensions were obtained from macroscopically healthy human lung tissue as previously described (13). Briefly, human lung tissue was cut into small pieces and enzymatically digested for 45 min with DNase I and collagenase. Thereafter, the tissue was mechanically disrupted by plunging through a syringe, the cells were washed, and debris was removed by 30% Percoll centrifugation. After preparation, the cells were stained and analyzed by flow cytometry.

Flow Cytometry

The following antibodies were used: anti-CD45-V500 (Clone HI30, BD Biosciences, San Jose, CA, USA), anti-CD14-APC-Cy7 (clone M5E2, BioLegend, San Diego, CA, USA), anti-CD117-APC (clone 104D2, BD Biosciences), anti-FcεRI-FITC (clone CRA1, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-FcεRI-PE (clone CRA1, BioLegend), anti-SUSD2-PE (clone W3D5, BioLegend), anti-CD63-FITC (clone H5C6, BD Biosciences), anti-CD49a-BV786 (clone SR84, BD Biosciences), anti-CD66a/c/e-A488 (clone ASL-32, BioLegend), anti-CD326-BV650 (clone 9C4, BioLegend), anti-CD34-BV421 (clone 581, BD Biosciences), anti-HLA-DR-PE/Cy5 (clone L243, BioLegend), anti-CD344-PE/Vio770 (clone CH3A4A7, Miltenyi Biotec), anti-CD38-BV421 (clone HIT2, BD Biosciences), anti-tryptase (clone G3, Millipore, Burlington, MA, USA) conjugated in-house with an Alexa Fluor 647 monoclonal antibody labeling kit (Thermo Fisher Scientific, Waltham, MA, USA), anti-CPA3 antibodies (clone CA5, a kind gift from Andrew Walls, Southampton, UK) conjugated in-house with an Alexa FluorTM 488 antibody labeling kit (Thermo Fisher Scientific) and chymase (clone B7, Millipore) conjugated in-house with a PE Conjugation Kit (Abcam, Cambridge, UK). Surface staining was performed by incubation for 30 min at 4 degrees with the antibodies in PBS+ 2% FBS, followed by washing with PBS+ 2% FBS. When using the LEGENDScreenTM human cell screening kit, which contains 332 markers and 10 isotype controls, conjugated to PE (Cat. 70001, BioLegend) that are detailed in **Supplementary Table S1**, cells were first stained with anti-CD45, anti-CD117, anti-CD14 and anti-FcεRI antibodies for 30 min, followed by washing. Thereafter, the cells were stained with the kit reagents according to the manufacturer's instructions. The cells from each donor were not sufficient to run an entire screen. Therefore, each kit was run using several donors, and a total of 10 donors were used to run three Legendscreens kits. The presence of anti-FcεRI antibody in the backbone panel did not result in mast cell activation, as measured by surface CD63 expression (data not shown). For intracellular staining, cells were fixed with 4% paraformaldehyde (PFA) and permeabilized using 0.1% saponin in PBS with 0.01 M HEPES (PBS-S buffer). Nonspecific binding was blocked using blocking buffer (PBS-S with 5% dry milk and 2% fetal calf serum (FCS)). The cells were thereafter

stained intracellularly over-night with the antibodies in blocking buffer, thereafter washed with PBS-S buffer and PBS+ 2% FBS. The cells were analyzed using a BD FACSCanto (BD, Franklin Lakes, NJ, USA) or BD LSRFortessa, and FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA) was used for flow cytometry data analysis. Fluorescent minus one (FMO) controls (extracellular) or isotype controls (intracellular) were used to establish gates for positive staining (**Supplementary Figure S1**).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software version 7.0b (**Figures 1C, 2 and 4**) or the Python environment (3.7) with the following packages: statsmodels (0.10.1), seaborn (0.9.0), scipy (1.4.1), pandas (1.1.0), numpy (1.18.1), and matplotlib (3.1.3) (**Figure 1D**). The specific methods used are detailed in the figure legends. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

RESULTS

Expression of Mast Cell Proteases

The classic division into distinct human mast cell subpopulations is based on the presence or absence of granule-associated antigens, i.e., the proteases tryptase, chymase and CPA3. We therefore first performed intracellular staining of lung cells and used a flow cytometry-based readout to accurately characterize the mast cell population with respect to the expression of tryptase, chymase and CPA3. Flow cytometry analysis identified CD45⁺CD14^{low}CD117^{high} HLMCs with the characteristic expression of tryptase (**Figures 1A, B**). The HLMC population showed a high variation of chymase and CPA3 expression between individual cells and the degree of CPA3 and chymase double positive cells varied considerably from donor to donor (**Figure 1C**). However, no distinct subpopulations were discernable, instead there was a continuous spectrum of expression levels.

Immunoprofiling of HLMCs

Whereas intracellular antigens failed to discriminate distinct subpopulations, we set out to perform an extensive mapping of cell surface antigens on HLMCs to potentially identify distinct subpopulations and novel markers. Flow cytometry analysis characterized the expression of 332 surface markers on HLMCs, using a LEGENDScreenTM human cell screening kit. Since many of the markers are broadly expressed we used CD14⁺ cells as a reference to selectively enrich for lineage-specific antigens in HLMCs (**Figure 1D**). Well-known monocyte markers such as CD11b, CD11c, and HLA-DR were highly expressed on CD14⁺ cells, verifying the validity in enriching for lineage-characteristic markers. In analogy, HLMCs expressed high levels of CD117 and FcεRI. Markers with the most significant differences between the HLMCs and CD14⁺ cells included CD9, CD59, CD274 and CD226 (**Figure 1D**). CD9 is a broadly expressed tetraspanin with a wide variety of functions; in mast cells, it is abundantly

expressed and has been implicated in chemotaxis and activation (14). CD59 can prevent complement-induced cytolytic cell death by preventing assembly of the complement membrane attack complex and has also been implicated in T cell activation (15). CD274 is also known as programmed death ligand-1 (PD-L1) and can cause blockade of T cell activation (16). CD226 has received increasing interest in recent years and can play a role in many immunological processes (17), including enhancement of FcεRI-mediated activation in mast cells (18).

Of the 332 markers analyzed, HLMCs showed significant expression of 102 markers (**Figure 2**), of which 23, to the best of our knowledge, have not been described on (non-neoplastic) human MCs before (**Table 1**).

Heterogeneous Expression of the High-Affinity IgE Receptor FcεRI

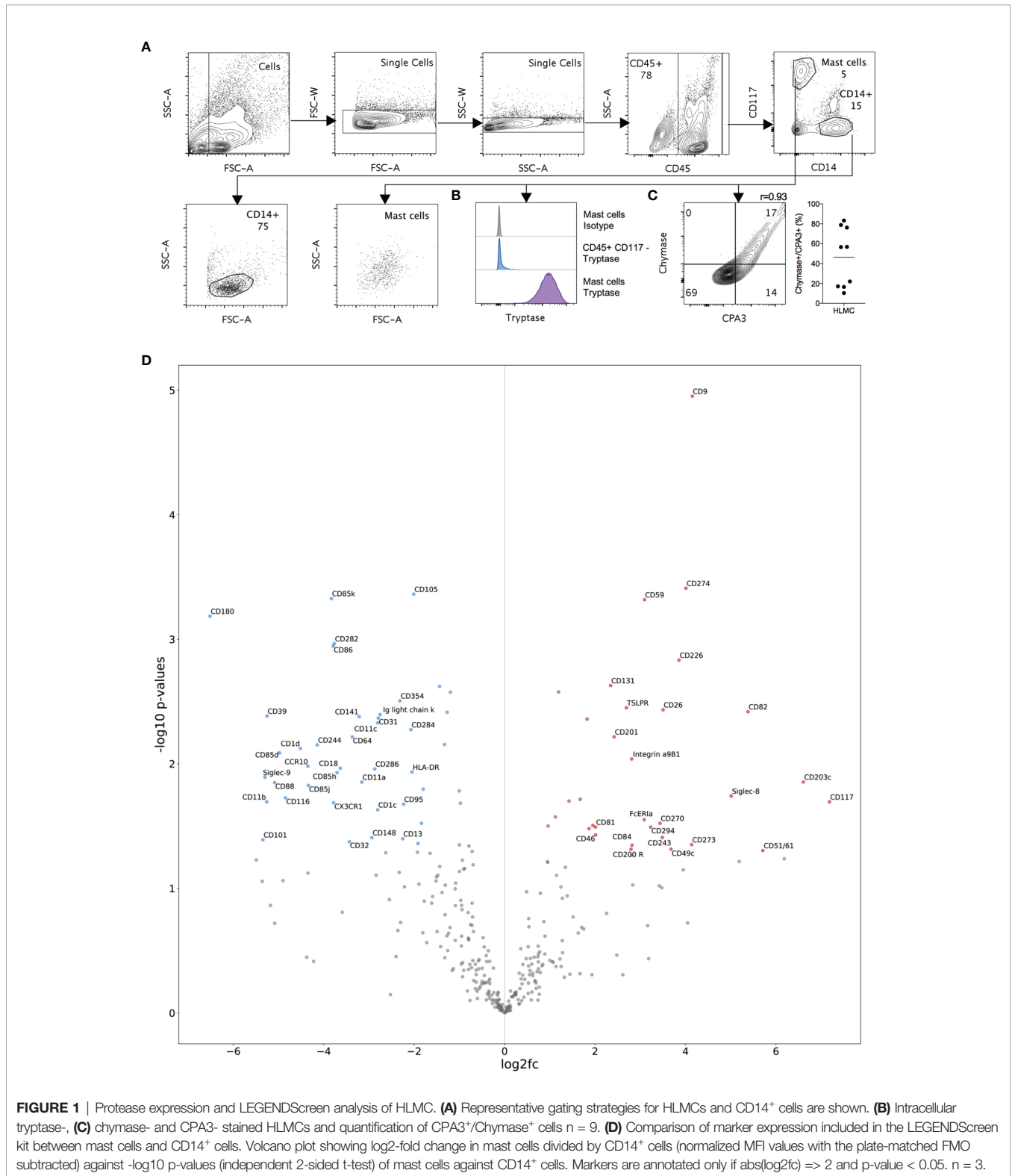
One of the principal markers for MCs is the expression of the high-affinity IgE-receptor FcεRI. FcεRI expression on HLMCs has been shown to differ among compartments in the lungs, as well as between healthy and diseased tissues. HLMCs from healthy individuals present in the parenchyma are negative for FcεRI, while patients with concurrent asthma show higher expression of the receptor (12, 19). In our study, the expression varied considerably between donors and there was no clear separation between the negative and positive FcεRI populations but rather a continuous spectrum of expression and approximately 50% of the donors expressed FcεRI on virtually all MCs (**Figures 3A–C**).

Heterogeneous Expression of Cell-Surface Markers With a Continuous Distribution

The heterogeneity of HLMCs has primarily been studied using immunohistochemistry in a binary manner and they have been divided into the MC_T and MC_{TC} subtypes (2, 3). How this heterogeneity is reflected by the heterogeneous expression of cell-surface markers has barely been investigated. None of the surface markers investigated in this study, using quantitative flow cytometry, did distinctly and consistently divide the HLMCs into subpopulations (data not shown). However, several markers were expressed with a considerable continuous variation within the HLMC population (**Table 2**). Co-stainings of nine of these markers revealed that expression of six of the markers, SUSD2, CD49a, CD326, CD34, CD66 and HLA-DR correlated ($r > 0.4$) (**Figures 4A–H**). The correlations seen between markers was not due to autofluorescence since there was no correlations in the FMO controls (**Supplementary Figures S1A–E**). In addition, the HLMCs showed no significant staining with the 10 isotype controls (**Figure 2**).

We next investigated if the expression of these markers correlated to the classical mast cell subsets using intracellular staining for CPA3 as a marker for MC_{TC}. Expression of neither SUSD2, CD38 (**Figures 4I, J**) nor CD344 (data not shown) correlated with CPA3 expression.

Since the expression of SUSD2, a marker for stem/progenitor cells (20), correlated to CD34, that is expressed on circulating



mast cell progenitors (21), we investigated if the expression of these markers could identify MCs in different stages of maturation. When mast cells mature they become larger with an increasing number of granules (22). We therefore gated

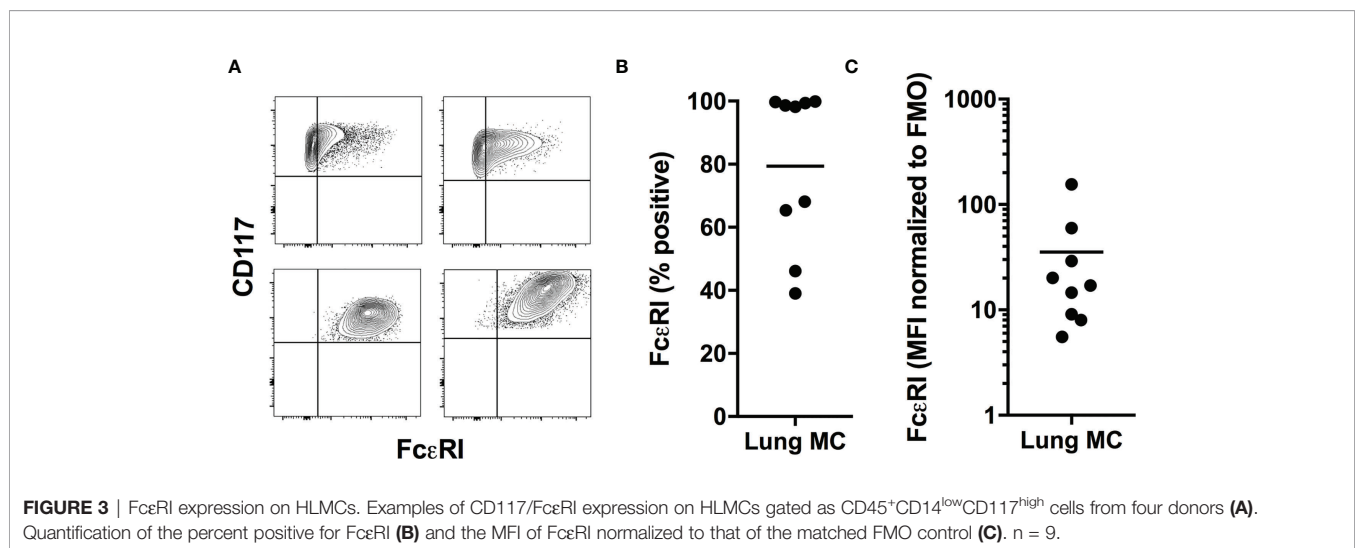
SUSD2 low, intermediate and high cells and compared the FSC and SSC values that reflect the size and granularity of the cells. However, SUSD2^{high} cells had higher FSC and SSC than SUSD2^{low} cells (**Figures 4K–M**), suggesting that they were

Marker	%	MFI	Marker	%	MFI	Marker	%	MFI	Marker	%	MFI			
CD1a	6	1	CD82	100	10	****	CD184	4	0	CCR10	30	1		
CD1b	8	1	CD83	56	3	***	CD193	2	0	CLEC12A	92	3	***	
CD1c	5	1	CD84	100	7	****	CD195	4	0	CLEC9A	2	0		
CD1d	8	1	CD85a	12	1		CD196	4	0	CX3CR1	1	0		
CD2	52	2	CD85d	10	1		CD197	3	0	CXCR7	2	0		
CD3	25	1	CD85g	5	1		CD200	6	1	OPRD	2	0		
CD4	76	3	CD85h	13	1		CD200R	100	5	****	DLL1	3	0	
CD5	9	1	CD85j	34	2		CD201	36	1	*	DLL4	1	0	
CD6	12	1	CD85k	26	1		CD202b	2	0	DR3	14	1		
CD7	22	1	CD86	9	1		CD203c	98	4	****	EGFR	4	0	
CD8a	16	1	CD87	12	1		CD205	39	1	*	erbB3	1	0	
CD9	100	11	CD88	9	1		CD206	5	1		FcεR1α	41	2	
CD10	53	3	CD89	8	0		CD207	2	0		FcRL6	2	0	
CD11a	73	4	CD90	17	1		CD209	1	0		Galectin-9	4	0	
CD11b	51	3	CD93	5	0		CD210	3	0		GARP	3	0	
CD11b act	31	1	CD94	2	0		CD213a2	3	0		HLA-A,B,C	99	7	****
CD11c	85	4	CD95	28	1	*	CD215	19	1		HLA-A2	35	3	*
CD13	97	5	CD96	3	0		CD218a	4	0		HLA-DQ	46	2	****
CD14	26	1	CD97	100	5	****	CD220	2	0		HLA-DR	84	4	****
CD15	3	0	CD99	100	6	****	CD221	8	1		HLA-E	38	2	****
CD16	35	2	CD100	32	2	*	CD226	88	3	****	HLA-G	9	1	
CD18	94	5	CD101	3	0		CD229	1	0		IFNGR2	2	0	
CD19	2	0	CD102	26	1		CD231	7	1		Ig light chain κ	26	1	
CD20	5	0	CD103	1	0		CD235ab	4	0		Ig light chain λ	36	2	
CD21	10	1	CD104	6	0		CD243	77	3	**	IgD	1	0	
CD22	99	5	CD105	5	1		CD244	2	0		IgM	1	0	
CD23	2	0	CD106	2	0		CD245	18	1		IL-28RA	2	0	
CD24	21	1	CD107a	80	3	****	CD252	3	0		Integrin α9β1	77	3	****
CD25	8	0	CD108	6	1		CD253	2	0		Integrin β5	7	1	*
CD26	92	5	CD109	2	0		CD254	1	0		Integrin β7	11	1	***
CD27	3	0	CD111	30	1	*	CD255	2	0		Jagged 2	6	1	
CD28	31	1	CD112	13	1		CD257	7	1		LAP	1	0	
CD29	100	7	CD114	5	1		CD258	3	0		LT-BR	8	1	****
CD30	35	2	CD115	40	2	***	CD261	2	0		Mac-2	3	0	
CD31	74	4	CD116	6	0		CD262	5	0		MAIR-II	11	1	****
CD32	56	3	CD117	100	5	****	CD263	2	0		MICA/MICB	1	0	
CD33	100	7	CD119	55	2	***	CD266	2	0		SUSD2 (W3D5)	45	2	****
CD34	58	2	CD122	1	0		CD267	2	0		SUSD2 (W5C5)	46	2	****
CD35	22	1	CD123	8	1		CD268	1	0		W7C6	2	0	
CD36	68	4	CD124	5	1		CD270	100	5	****	W4A5	53	2	****
CD38	68	4	CD126	6	1		CD271	2	0		MSCA-1	1	0	
CD39	41	2	CD127	2	0		CD273	63	2	*	NKp80	0	0	
CD40	33	2	CD129	4	0		CD274	93	3	***	Notch 1	2	1	
CD41	58	3	CD131	65	2	****	CD275	17	1		Notch 2	2	1	
CD42b	2	0	CD132	17	1		CD276	93	4	****	Notch 3	1	0	
CD43	100	6	CD134	4	0		CD277	91	3	***	Notch 4	1	0	
CD44	100	11	CD135	2	0		CD278	2	0		NPC (57D2)	3	0	
CD45	92	5	CD137	4	0		CD279	1	0		Podoplanin	7	1	
CD45RA	86	4	CD137L	12	1		CD282	1	0		Pre-BCR	2	0	
CD45RB	40	2	CD138	18	1		CD284	2	0		PSMA	6	0	
CD45RO	90	5	CD140a	2	0		CD286	2	0		Siglec-10	25	2	****
CD46	100	8	CD140b	5	1		CD290	1	0		Siglec-8	82	3	****
CD47	100	9	CD141	25	1		CD294	24	1		Siglec-9	18	1	****
CD48	99	6	CD143	33	1	*	CD298	100	7	****	SSEA-1	1	0	
CD49a	47	2	CD144	3	0		CD300e	28	1		SSEA-3	1	0	
CD49c	99	6	CD146	23	1		CD300F	96	4	****	SSEA-4	1	0	
CD49d	97	5	CD148	56	2	***	CD301	4	1		SSEA-5	22	1	****
CD49e	92	5	CD150	2	0		CD303	2	0		TCR γ/ε	3	0	
CD49f	54	2	CD152	20	1		CD304	30	1		TCR Vβ13.2	1	0	
CD50	81	4	CD154	5	0		CD307	2	0		TCR Vβ23	2	0	
CD51	100	6	CD155	26	1	*	CD307d	2	0		TCR Vβ8	1	0	
CD51/61	100	6	CD156c	94	4	****	CD314	3	0		TCR Vβ9	0	0	
CD52	92	5	CD158a/h	2	0		CD317	95	3	****	TCR Vα2	1	0	
CD53	98	5	CD158b	5	0		CD318	13	1		TCR Vγ9	1	0	
CD54	99	6	CD158d	34	1		CD319	5	1		TCR Vα24-Jα18	6	1	
CD55	100	6	CD158e1	2	0		CD324	3	1		TCR Vα7.2	1	0	
CD56	3	0	CD158f	2	0		CD325	2	0		TCR α/β	2	0	
CD57	3	0	CD161	2	0		CD326	45	2		Tim-1	1	0	
CD58	100	7	CD162	92	4	****	CD328	65	3	**	Tim-3	30	2	****
CD59	100	10	CD163	18	1		CD334	1	0		Tim-4	1	0	
CD61	90	4	CD164	100	5	****	CD335	5	0		TLT-2	0	0	
CD62E	5	1	CD165	2	0		CD336	1	0		TRA-1-60-R	1	0	
CD62L	6	0	CD166	88	3	****	CD337	2	0		TRA-1-81	1	0	
CD62P	35	2	CD167a	13	1		CD338	1	0		TSLPR	10	1	****
CD63	100	7	CD169	2	0		CD340	1	0		Ms IgG1 κ	1	0	
CD64	55	3	CD170	71	2	****	CD344	55	2	*	Ms IgG2a κ	4	1	
CD66a/c/e	67	3	CD172a	74	3	****	CD351	2	0		Ms IgG2b κ	1	0	
CD66b	4	0	CD172b	11	1		CD352	12	1		Ms IgG3 κ	2	0	
CD69	99	6	CD172g	5	1		CD354	15	1		Ms IgM κ	0	0	
CD70	5	1	CD178	33	1		CD355	2	0		Rat IgG1	1	0	
CD71	66	3	CD179a	9	1		CD357	4	1		Rat IgG2a	2	0	
CD73	42	2	CD179b	2	0		CD360	2	0		Rat IgG2b	1	0	
CD74	55	3	CD180	1	0		B2M	100	9	****	Rat IgM κ	0	0	
CD79b	25	1	CD181	6	0		BTLA	2	0		AH IgG	1	0	
CD80	18	1	CD182	1	0		C3AR	50	2					
CD81	100	8	CD183	7	0		CSL2	5	1					

FIGURE 2 | Expression of cell-surface antigens on HLMCs. HLMCs stained with the LEGENDScreen human cell screening kit. Mast cells were gated as CD45⁺CD14^{low}CD117^{high} cells. Shown are the percent positive (%) for each marker, the MFI (normalized to the plate-matched FMO control and log₁₀ transformed) and the stars represents the significance of the MFI of the marker compared to that of the FMO control (one-way ANOVA with Dunnett's multiple comparisons test). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. n = 3.

TABLE 1 | Novel antigens identified on HLMCs.

Marker (clone)	Description
CD36	Receptor binding a broad range of lipids
CD45RO	Isoform of CD45
CD66a/c/e	Adhesion molecules
CD74	Involved in MHC class II antigen processing and a receptor for macrophage migration inhibitory factor
CD111	Adhesion molecule
CD115	Receptor for M-CSF and IL-34
CD131	Common β subunit of the IL-3, IL-5 and GM-CSF receptors
CD143	Metallopeptidase
CD148	Tyrosine phosphatase involved in signal transduction
CD164	Sialomucin involved in cell adhesion and proliferation
CD166	Glycoprotein involved in cell adhesion and migration
CD205	Endocytic receptor involved in antigen uptake and processing
CD243	Involved in transportation of molecules across cell membranes
CD270	Receptor for TNFSF14, BTLA, LTA and CD160
CD277	Regulate T cell responses
CD317	Blocks the release of certain viruses from infected cells
CD344 (Frizzled-4)	Receptor for Wnt proteins and norrin
CLEC12A/CD371	C-type lectin-like receptor with an immunoreceptor tyrosine-based inhibitory motif (ITIM) domain
Integrin $\alpha 9\beta 1$	Integrin mediating cell adhesion and migration
SUSD2 (W3D5, W5C5) (W4A5)	Potentially involved in cell adhesion as this transmembrane protein contains functional domains associated with adhesion molecules
Siglec-9	Antigen has yet to be described
SSEA-5	Lectin that binds sialic acid and has ITIM domains
	A glycan



larger with a higher granularity and therefore unlikely to be immature MCs.

SUSD2 has been linked to proliferation in cancer cells (23), why we investigated the proliferation status of the cells with the proliferation marker Ki-67. However, in agreement with the fact that mast cells are long-lived cells with low turnover (24), no staining was observed (Supplementary Figure S2).

DISCUSSION

HLMCs have been shown to be heterogeneous; classically, they have been studied using immunohistochemistry in a binary manner, and they have been divided into the MC_T and MC_{TC}

subtypes based on whether the mast cell proteases chymase and CPA3 are detectable (2, 3). By using a quantitative flow cytometry based method we indeed found high variation in chymase and CPA3 expression but no distinct subpopulations were discernable (Figure 1C). The apparent discrepancy between our study and previous immunohistochemical studies is probably due to the fact that we have studied the expression in a quantitative manner using flow cytometry, thus finding that there is a spectrum of different expression levels. In contrast, in previous studies the cells have been classified into MC_T/MC_{TC} cells in a binary manner depending on the detection limit of the immunohistochemical technique.

Although attempts have been made to map cell-surface antigens on HLMCs (25–30), extensive mapping including the

TABLE 2 | The 10 markers from the LEGENDScreen analysis with the highest robust coefficient of variation (robust CV).

Marker	Robust CV
SUSD2 (W5C5)	264
SUSD2 (W3D5)	246
CD344	172
CD49a	160
CD326	155
CD66a/c/e	153
CD34	134
HLA-DR	133
SSEA-5	131
CD63	124
CD38	123

heterogeneity of cell-surface antigen expression has not been carried out. In this study, we identified significant expression of 102 markers on the HLMC surface (Figure 2), of which, to the best of our knowledge, 23 are novel mast cell markers (Table 1). Several of these markers, including SSEA-5, SUSD2, W4A5, CD243, CD111, CD131 and CD164, are described as markers expressed on stem cells. The expression of stem cell markers on mast cells is in accordance with results from the FANTOM5 consortium, in which skin mast cells exhibited similarities with stem cells (31). In some cases, our results are in disagreement with previously published data; for example, CD4, CD10, CD36 and CD74 were previously shown to not be expressed by HLMCs (26, 27). This discrepancy might be explained by differences in the procedures, as in contrast to published data, we did not purify or culture the studied mast cells prior to analysis (25, 27, 29, 30). Culturing mast cells has been shown to alter their phenotype and expression of cell-surface receptors (31, 32). Furthermore, although macroscopically healthy tissue distal from the tumor was used for our analysis, one cannot rule out the possibility that also this part of the tissue is affected by the disease and can have an impact on the results.

The expression of FcεRI has been shown to differ in different compartments of the lung, with mast cells present in the parenchyma being negative for FcεRI (12). We have investigated single cell suspensions from lung tissues without separation of the parenchymal cells. However, we did not observe distinct FcεRI positive and negative mast cell populations but rather a continuous spectrum of expression levels (Figure 3A). Additionally, in about 50% of the donors virtually all mast cells stained positive for FcεRI, meaning that we cannot detect any FcεRI negative parenchymal mast cell population in these individuals (Figure 3A). This discrepancy is again likely to be due to the different techniques used, immunohistochemistry and flow cytometry, and the different detection limits of the techniques. We also observed a large variation in expression of FcεRI among individuals (Figures 3A–C), and in line with our results, this has previously been shown also for human skin mast cells (33). The reasons for this variation could be manifold, as the surface expression of FcεRI can be regulated in many different ways. FcεRI is, for example, upregulated by IL-4 and stabilized on the cell surface by the

binding of IgE antibodies (34). Furthermore, it was described recently that IL-33 downregulates the expression of FcεRI (35, 36), indicating that the state of inflammation in the tissue can influence FcεRI expression.

Heterogeneous expression of cell-surface markers on mast cells has scarcely been investigated. We investigated the heterogeneity of cell-surface markers in a quantitative manner using flow cytometry and did not find any markers that distinctly and consistently divided the studied mast cells into subpopulations with a bi- or multimodal distribution (data not shown). We did, however, find several markers with considerable continuous variation in expression within the mast cell populations (Table 2), and co-stainings revealed that six of these markers, SUSD2, CD49a, CD326, CD34, CD66 and HLA-DR, were correlated (Figure 4). To investigate whether these markers are correlated with the classic mast cell subpopulations MC_T and MC_{TC}, we costained for SUSD2 and CPA3, but no correlation was detected, ruling out the possibility that these markers are extracellular markers of the classic mast cell subtypes (Figure 4I). Recently, CD38 was demonstrated to be differentially expressed in human nasal polyp mast cells where CD38^{low} were of the MC_{TC} subtype, while CD38^{high} MCs were a heterogeneous pool of both MC_T and MC_{TC} cells (37). In our study, CD38 did not distinctly separate the HLMCs into subpopulations and did not correlate with CPA3; i.e., in HLMCs CD38 could not be used to distinguish the MC_T and MC_{TC} subtypes (Figure 4J). CD344 did not correlate with the MC_T or MC_{TC} profile either (data not shown). Another marker suggested to be expressed on MC_{TC} is the complement 5a receptor CD88 (38). However, we did not detect significant expression of CD88 on HLMCs (Figure 2). Thus, we were unable to find an extracellular marker that distinguishes the classical mast cell subsets.

One of the markers that showed high continuous variation in the HLMCs, CD63, is used as a surrogate marker for mast cell activation (39), i.e., degranulation. However, CD63 did not correlate to any of the other eight markers investigated, including the 6 markers that show correlation to each other SUSD2, CD49a, CD326, CD34, CD66 or HLA-DR indicating that these markers do not reflect varying degree of activation (Figure 4G and data not shown).

Since two of our six heterogeneity markers that correlate, CD34 and SUSD2, is expressed on stem/progenitor cells (20, 21, 40), we wondered whether these markers could identify cells in different stages of mast cell maturation. However, if that was the case, one would expect cells with high expression of SUSD2/CD34 to be small and contain few granules similar to mast cell progenitors (21). In contrast, the cells with high expression of SUSD2 had relatively high FSC and SSC values (Figures 4K–M), suggesting that they were relatively large and granular and therefore unlikely to be immature mast cells. In this context, it is worth noting that mature murine mast cells also express CD34 (41), and in these cells, CD34 inhibits adhesion and is required for optimal migration (42).

SUSD2 is also expressed in certain cancers, in which it has been linked to proliferation (23); thus, one could imagine that

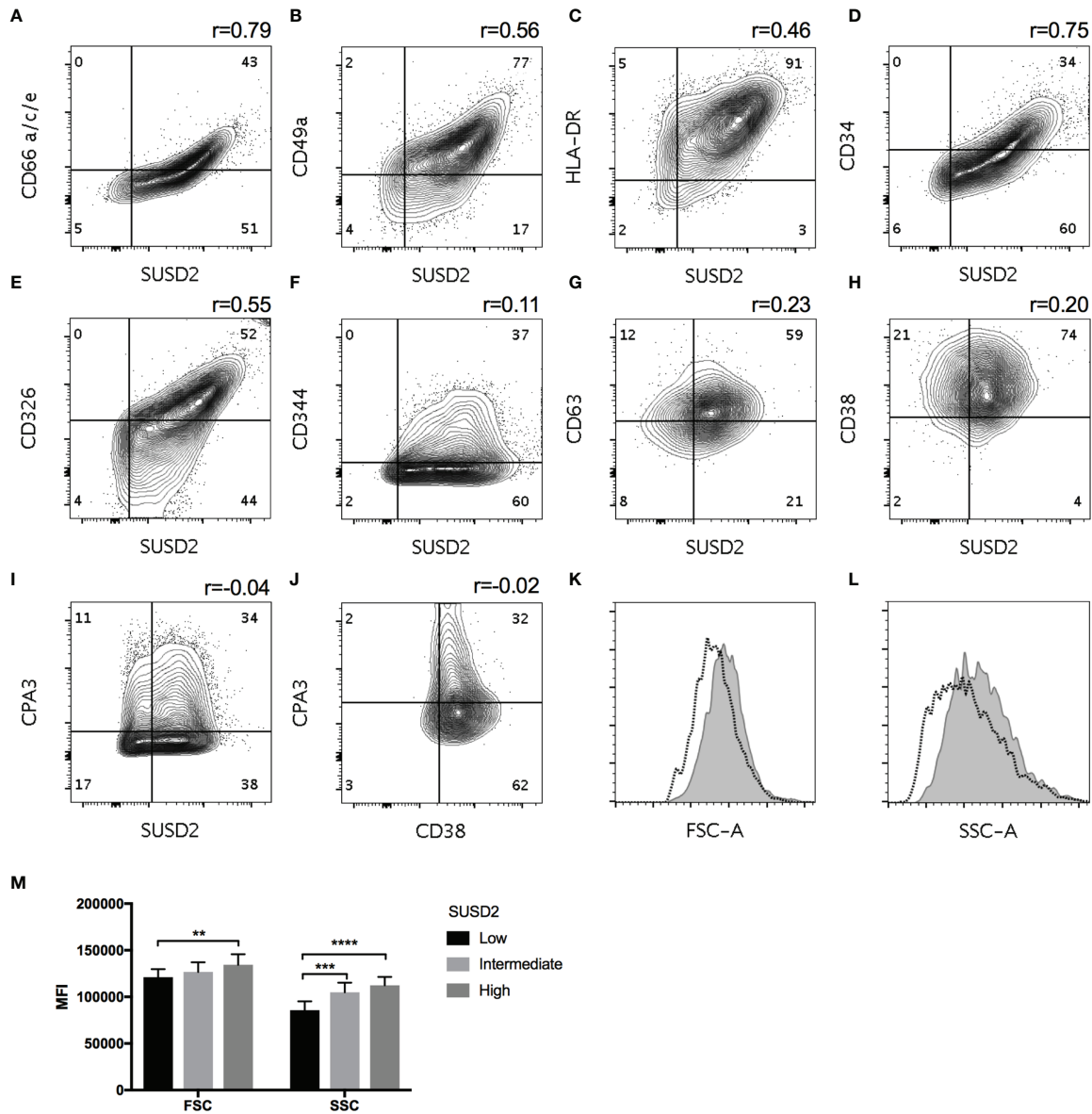


FIGURE 4 | Co-staining of HLMCs with indicated markers. Pearson correlations in each donor was calculated and the average r is shown ($n = 3-4$) (A–J). SUSD2low, intermediate and high cells were gated, and the FSC (K) and SSC (L) values of the SUSD2low (dotted line) and SUSD2high (filled gray) populations are shown. Quantification of FSC and SSC is shown in (M), mean \pm SEM, $n = 5$. Two-way ANOVA with Bonferroni's multiple comparisons test was performed. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

cells with high SUSD2 expression are proliferating. However, we could not detect any staining for the proliferation marker Ki67 in HLMCs (Supplementary Figure S2).

In summary, we have identified the expression of 102 cell-surface antigens on HLMCs, of which 23 have not been described previously on MCs. Several of these antigens had a high continuous variability in their expression within the HLMC population. The expression of six of these markers correlated to each other and the size and granularity of the cells. Further studies are needed to determine how these cells differ functionally. None of the markers correlated with the

intracellular protease expression. Thus, in contrast to the dogma of distinct mast cell subtypes, we demonstrate the continuous nature of HLMC heterogeneity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regionala Etikprövningsnämnden, Stockholm. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ER, JD, and GN conceived and designed the studies. ER, JD, and AR designed and performed the experiments. ER, DZ, and JD analyzed the data. JS, A-CO, MA-A, MA, and S-ED provided samples. ER, GN, and JD wrote the manuscript draft. All authors reviewed, critically revised, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.804812/full#supplementary-material>

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