Altered leukocyte subsets and immune proteome indicate proinflammatory mechanisms in mastocytosis

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Background: Indolent systemic mastocytosis (ISM) is characterized by pathologic accumulation of mast cells. The mechanism behind its phenotypic heterogeneity is not well understood. Interaction of mast cells with other immune cells might cause systemic inflammation and thereby associated symptoms.
Objective: We investigated peripheral leukocyte compartments and serum immune proteome in ISM.
Methods: Peripheral blood leukocyte phenotyping using flow cytometry in a cohort of 18 adults with ISM and 12 healthy controls. Targeted proteomics was performed to measure 169 proteins associated with inflammation on serum of another 20 ISM patients and 20 healthy controls.
Results: Proportions of plasmacytoid dendritic cells and monocytes were significantly decreased while T_H2 cells were increased in peripheral blood of ISM patients. Furthermore, a shift from naive to memory T cells was observed. Hierarchical clustering of the serum proteome revealed 2 distinct subgroups within ISM patients. In subgroup A (n = 8), 62 proteins were significantly overexpressed, whereas those of subgroup B (n = 12) were comparable to healthy controls. Patients in subgroup A displayed upregulated signaling pathways downstream of Toll-like receptor 4, TNF-α, and IFN-γ. Fatigue was more often present in subgroup A compared to B (75% vs 33% respectively, $P = .06$).
Conclusions: Altered distribution of leukocyte subsets and a proinflammatory proteome were observed in subsequent 2 cohorts of ISM patients. We hypothesize that neoplastic mast cells recruit and activate plasmacytoid dendritic cells, monocytes, and T cells, leading to a vicious cycle of inflammation. (J Allergy Clin Immunol 2022;150:146-56.)

Key words: *Mastocytosis, proteomics, mast cell, dendritic cell, monocyte, T cell, IL-6, TNF-α, interferon*

Systemic mastocytosis is a rare hematologic disease caused by clonal expansion of mast cells. This is most often associated with a somatic mutation in the *KIT* gene, leading to autonomous tyrosine kinase activity of the KIT receptor[1] and resultant increased proliferation and survival of clonal mast cells. According to World Health Organization criteria, the diagnosis of SM can be made when accumulation of abnormal mast cells is found in at least 1 extracutaneous organ, most often the bone marrow.[2]

Indolent systemic mastocytosis (ISM) is the most prevalent form of systemic mastocytosis. Although patients have a normal life expectancy, they may experience a wide range of unpleasant symptoms, including itch, flushing, anaphylaxis, dyspepsia, diarrhea, and osteoporosis.[3] Nonspecific symptoms, such as fatigue and myalgia, are also often reported and can negatively influence the quality of life.[4-6] The heterogeneous clinical phenotype of mastocytosis, especially among patients with ISM, is still unexplained. It is generally assumed that symptoms are caused by excessive levels of mast cell–derived mediators, including histamine, proteases, eicosanoids, chemokines, and cytokines.[7] However, although serum levels of tryptase, histamine, and prostaglandins generally correlate with mast cell burden in bone marrow, they show no clear association with the clinical phenotype of patients.[8,9] Likewise, serum levels of IL-6, soluble CD117, or soluble CD25 correlate with mast cell burden and the risk of disease progression but not with daily symptoms.[10,11] Nevertheless, the increased levels of these proinflammatory cytokines in the circulation suggest that immune activation beyond mast cell activity is likely to be involved in mastocytosis.

Mast cells are preferentially located in the skin and mucosa, where they are able to communicate with neighboring cells, including dendritic cells, macrophages, lymphocytes, epithelial cells, and fibroblasts.[12] Interestingly, ISM patients without skin involvement more often experience anaphylaxis and osteoporosis, but less often have typical mast cell mediator–related symptoms than patients with skin involvement.[13] This suggests that pathophysiologic mechanisms might differ between patients with or without skin infiltration by neoplastic mast cells. Furthermore, we recently demonstrated the presence of increased innate

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Abbreviations used
CCL: C-C motif chemokine ligand
IPA: Ingenuity Pathway Analysis
ISM: Indolent systemic mastocytosis
MCP-1: Monocyte chemoattractant protein 1
MPCM: Maculopapular cutaneous mastocytosis
NK: Natural killer
PCA: Principal component analysis
pDC: Plasmacytoid dendritic cells
TLR4: Toll-like receptor 4

lymphoid cells type 2 in peripheral blood of patients with ISM.[14] In particular, an association was found between the proportion of these type 2 cells and the presence of skin mastocytosis as well as itch, suggesting a proinflammatory skin environment in these patients.[14]

Together, these data indicate a broader immune involvement in mastocytosis, and involvement of different immune components may relate to unique clinical presentations. Although the skin is an obvious environment for the interaction between mast cells and other immune components, such interactions can theoretically occur at every mast cell–containing location. Widening our focus beyond mast cell involvement to include other elements of the immune system will provide more insight into the pathophysiology of mastocytosis, which may provide us with meaningful biomarkers—and ultimately targets for therapy.

The objective of this study was to gain better understanding of immune cells and inflammatory molecules in ISM. We conducted flow cytometric analysis of peripheral blood leukocyte subsets and targeted serum proteomics using 2 independent cohorts of adult mastocytosis patients.

METHODS

Participants

Two separate cohorts of adults with ISM were included in this study. First, cohort 1 was used for flow cytometric analysis of leukocyte subsets in peripheral blood cells of 18 adults with ISM and 12 healthy controls. The patients were prospectively included between November 2014 and May 2015 from the outpatient clinic of the mastocytosis center of Erasmus MC. Second, targeted proteomics analysis was performed on serum samples of 20 adults with ISM, forming cohort 2 (collected between April 2017 and March 2019, and stored at −80°C in the local biobank) and 20 age- and sex-matched healthy controls.

All patients fulfilled World Health Organization criteria for ISM.[2] Patients who were treated with immunosuppressive drugs including corticosteroids were excluded. Basic clinical characteristics for both cohorts were recorded at inclusion, and additional data were extracted from the electronic patient files. Two separate cohorts of healthy controls were recruited from hospital staff for each mastocytosis cohort.

Ethical considerations

This study was performed according to the latest Helsinki guidelines and was approved by the local medical ethics committee Erasmus MC, with protocol no. MEC-2014-443 for flow cytometry and nos. MEC-2019-0839 and MEC-2016-202 for proteomics. All participants provided informed consent.

Flow cytometry

Flow cytometric immunophenotyping of leukocyte subsets was performed as previously described.[15,16] In brief, fresh samples were prepared using a diagnostic lyse-no-wash protocol with commercial Trucount tubes (BD Biosciences, San Jose, Calif) for absolute counts of total T cells, B cells, and natural killer (NK) cells. Because mast cells are known to induce type 2 inflammation, for instance in allergy and helminth infections,[17,18] we hypothesized that an imbalance in T_H1 and T_H2 subsets might be present. T-helper cell subsets were therefore also analyzed. Detailed immunophenotyping of leukocyte subsets was conducted using 3 sets of antibody panels. The antibodies we used are listed in Table E1 in the Online Repository available at www.jacionline.org. The gating strategy for T-helper cell subsets is shown in Fig E1 in the Online Repository. Red blood cells were lysed with 150 mmol/L NH_4CL. Subsequently, 1 million nucleated cells per panel were incubated with antibodies for 15 minutes at room temperature in a total volume of 100 μL. Samples were measured on a 4-laser LSR Fortessa flow cytometer (BD Biosciences) using standardized settings. Immunophenotypic definitions of all leukocyte subsets are listed in Table E2 in the Online Repository. Via the validated Trucount method, absolute cell numbers were available for NK cells and T-cell and B-cell subsets. For the other leukocyte subsets (monocytes, granulocytes, neutrophils, eosinophils, basophils, and plasmacytoid dendritic cells, or pDC), percentages were deemed more reliable because these were not measured in Trucount tubes. Data were analyzed by FACSDiva v8 software (BD Biosciences).

Proteomics assay

A targeted proteomics assay was performed on serum using 2 prespecified panels of a total of 169 unique proteins involved in the immune response and inflammation. A list of the analytes is provided in Table E3 in the Online Repository available at www.jacionline.org. The technical analysis was outsourced to Olink Proteomics (Uppsala, Sweden). The data were provided as normalized protein expression, which serves as a surrogate for protein concentration.

Statistical analysis

SPSS v25.0 (IBM, Armonk, NY) was used for the statistical analysis of patient characteristics, and GraphPad Prism v6.0 (GraphPad Software, La Jolla, Calif) was used for the analysis of flow cytometry data. Continuous variables are provided as medians with interquartile ranges, and dichotomous variables as absolute numbers with percentages. Continuous variables were compared by the Mann-Whitney *U* test; dichotomous variables were compared by the chi-square test. The Spearman rho value was calculated for determining correlations between 2 continuous variables. All *P* values were 2 tailed and considered statistically significant if <.05.

Proteomics data were analyzed by Omniviz v6.1.13.0 (Instem Scientific, Staffordshire, UK) and Partek (Partek, St Louis, Mo). First, the overall data were explored via principal component analysis (PCA). Nonsupervised hierarchical clustering was performed, as well as volcano plotting based on ANOVA analysis. Second, the levels of protein expression of ISM patients were compared to the control group by significant analysis of microarray in Ingenuity Pathway Analysis (IPA; Qiagen, Germantown, Md) using a false discovery rate of 5% and fold change of ≥1.5. Pathway and upstream analyses were performed by IPA using a cutoff of ≥1.5 for fold change and a *z* score of ≥2 compared to healthy controls.

RESULTS

Patient characteristics

Cohort 1, used for flow cytometric immunophenotyping of peripheral blood leukocyte subsets, consisted of 18 ISM patients and 12 healthy controls (Table I). The median age of ISM patients was 52 years (range, 31-83 years), 12 subjects (67%) were female, and the median serum tryptase at the time of inclusion was 29 μg/L (range, 10.9-134 μg/L). Fifteen patients (83%) had skin

TABLE I. Baseline patient characteristics by cohort

Characteristic	Cohort 1 (flow cytometry) (n = 18)	Cohort 2 (proteomics) (n = 20)	*P* value*
Age (years), median (IQR)	52 (48-60)	48 (38-56)	.067
Male sex	6 (33)	6 (30)	.825
Serum tryptase (μg/L), median (IQR)	29.0 (20.5-74.3)	21.5 (13.4-35.8)	.067
D816V mutation detected†	10/12 (83)	14/18 (78)	.709
MPCM	15 (83)	14 (70)	.334
Itch	9 (50)	11 (55)	.404
Anaphylaxis	6 (33)	4 (20)	.351
Atopy	4 (22)	3 (15)	.687
Fatigue	6 (33)	10 (50)	.299
Diarrhea	4 (22)	9 (45)	.109
Decreased bone density	12/17 (70.6)	10 (50)	.204

Data are presented as nos. (%) unless otherwise indicated. Atopy indicates the presence of atopic dermatitis, allergic rhinoconjunctivitis, asthma, or specific IgE against inhalational allergens. The *t* score was measured by bone densitometry.
IQR, Interquartile range.
*Mann-Whitney *U* test for continuous variables and Fisher exact test for dichotomous variables.
†KIT D816V mutation status unknown in other patients.

involvement characterized as maculopapular cutaneous mastocytosis (MPCM).

Cohort 2, used for serum proteomics analysis, consisted of 20 patients with ISM and 20 healthy controls who were matched for age and sex. The median age of the ISM patients was 48 years (range, 21-63 years), 14 subjects (70%) were female, and the median serum tryptase at the moment of inclusion was 21.5 μg/L (range, 4.4-97 μg/L). Fourteen patients (70%) had MPCM. The patients of the 2 cohorts were overall comparable regarding demographic characteristics and mastocytosis-related symptoms (Table I). Individual patient characteristics are summarized in Table E4 in the Online Repository available at www.jacionline.org.

Leukocyte subsets

In cohort 1 (n = 18 adults with ISM), the proportions of total lymphocytes, total granulocytes, neutrophils, eosinophils, and basophils were comparable with healthy controls (Fig 1). However, the proportions of monocytes and pDC were significantly lower in patients with ISM ($P < .05$ and $P < .001$, respectively). While the total numbers of T-, B-, and NK cells were comparable between ISM and controls (data not shown), ISM patients displayed significantly higher numbers of T_H2 cells ($P < .05$), resulting in a decreased T_H1/T_H2 ratio, indicative of T_H2 skewing (Fig 2, *B*). Proportions of T_H17 cells, regulatory T cells, and follicular T-helper cells did not differ between patients and controls (data not shown). ISM was associated with increased numbers of $CD8^+$ central memory T cells ($P < .01$) and a relative decrease in $CD8^+$ naive T cells compared to healthy controls ($P \geq .05$, Fig 2, *C*). Although comparable skewing toward a memory phenotype was observed for $CD4^+$ T cells, this did not reach statistical significance (Fig 2, *D*).

There was no correlation between pDC proportions and the T_H1/T_H2 ratio or tryptase levels. Furthermore, the proportion of pDC did not correlate with the quantity of CD8 naive or memory subsets. Last, there was no difference in pDC percentage between patients with or without MPCM, itch, anaphylaxis, or fatigue (data not shown).

Distinct serum proteome profile in a subgroup of ISM patients

The serum proteome was investigated in a cohort 2, consisting of 20 patients with ISM. First, PCA was performed using the normalized protein expression values for all 169 investigated proteins (Fig 3). Eleven ISM patients localized near the healthy controls in the PCA plot, while 9 others were clearly different from the controls, 8 cases of which clustered together and 1 of which was a separate outlier.

Subsequent nonsupervised hierarchical clustering of all 169 investigated proteins on ISM and healthy control samples also revealed heterogeneity among ISM subjects (Fig 4, *A*), discerning between the same 2 ISM subgroups as seen in the PCA plot. Subgroup A (n = 8) displayed general upregulation of proinflammatory proteins compared to subgroup B (n = 12), which did not differ from the healthy control cohort, as visualized in the PCA plot (Fig 3). In fact, none of the measured proteins differed significantly between ISM subgroup B and healthy controls. We therefore continued with subgroup A for further analysis. Comparison of ISM subgroup A with healthy controls revealed 62 proteins to be significantly more highly expressed in this ISM subgroup (Fig 4, *B,* and see Fig E2 in the Online Repository available at www.jacionline.org). Although not statistically significant, there was a trend toward a higher prevalence of fatigue in ISM subgroup A compared to subgroup 2 (75% vs 33% respectively, $P = .06$). The 2 subgroups did not differ regarding other mastocytosis-related symptoms or atopy (Table II).

Upregulation of Toll-like receptor 4 signaling and IL-6 family cytokines in ISM subgroup A

IPA of the 62 proteins of interest in the serum of ISM subgroup A revealed 3 major pathways to be involved upstream of these proteins (see Table E5 in the Online Repository available at www.jacionline.org). This specifically concerned activation of the Toll-like receptor 4 (TLR4) pathway, with 28 of 62 proteins involved (*z* score, 4.192). Furthermore, 20 of 62 proteins were identified as downstream of TNF-α (*z* score, 4.043), and 18 of 62 proteins were downstream of IFN-γ (*z* score, 3.835).

Next, hierarchical clustering of the 62 significant proteins was conducted in order to identify coaggregation of clusters of proteins (see Fig E3 in the Online Repository available at www.jacionline.org). This revealed 5 clusters of coregulated proteins. These clusters were functionally analyzed by IPA and according

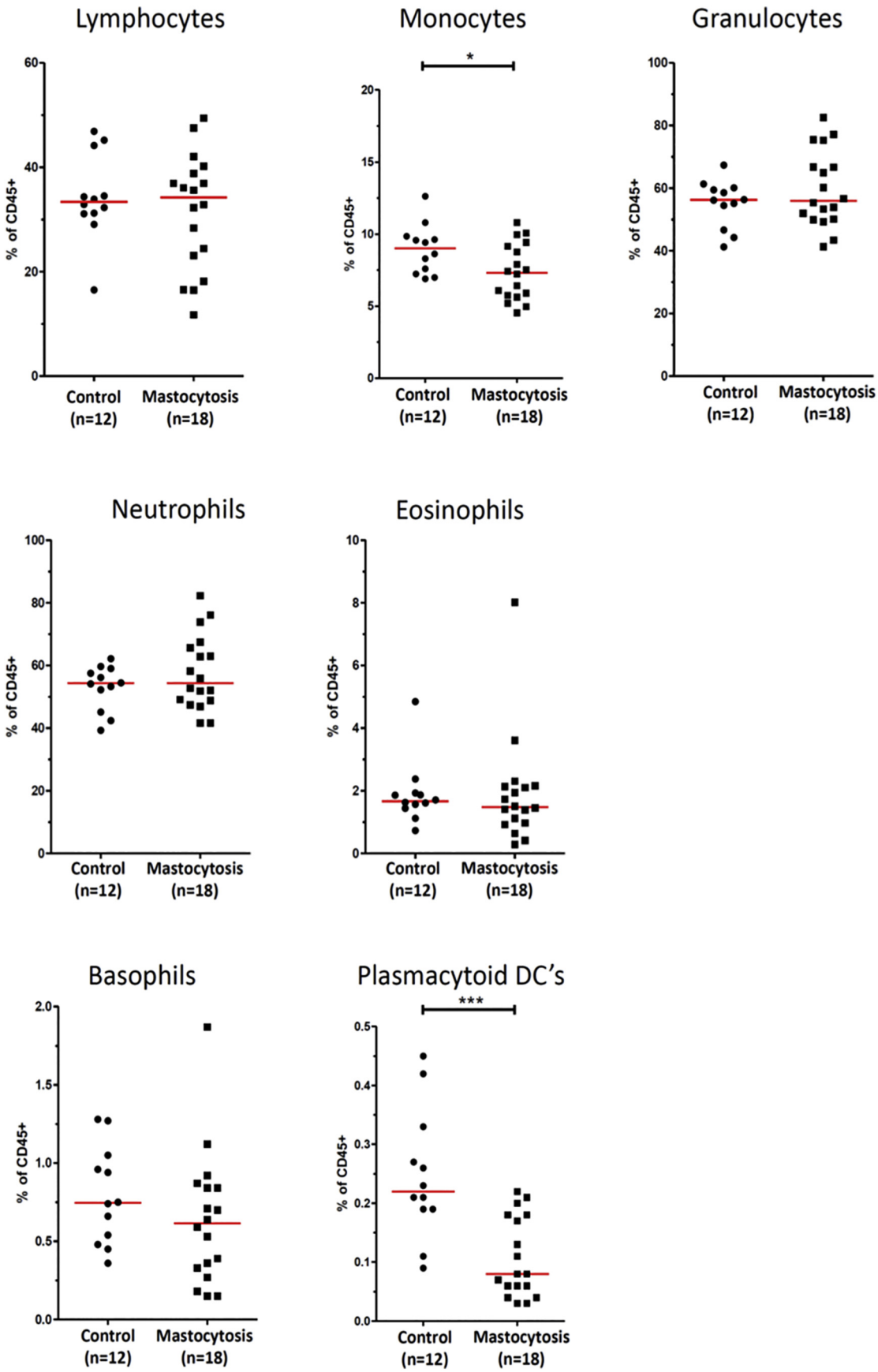

FIG 1. Peripheral blood leukocyte subsets in ISM (cohort 1). Flow cytometric analysis of leukocyte subsets in peripheral blood of patients with ISM compared to healthy controls revealed a decrease in the proportion of pDC, and to a lesser extent the monocyte proportion, in mastocytosis. *$P < .05$, ***$P < .001$.

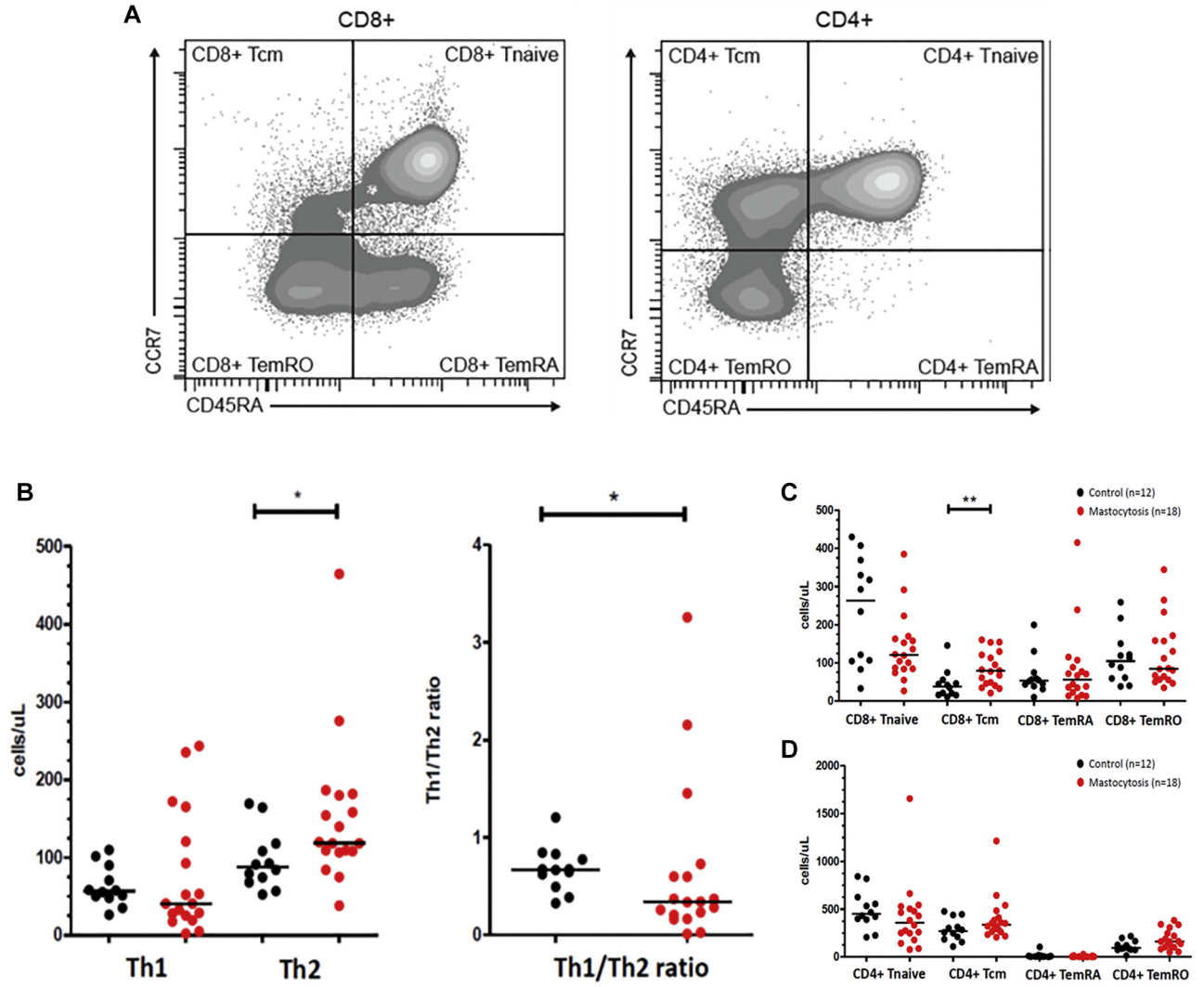

FIG 2. T-cell subsets in peripheral blood of ISM patients (cohort 1). **A**, Gating strategy of T-cell subsets. **B**, The total number of T_H2 cells was increased in blood. As a result, the T_H1/T_H2 ratio was decreased in ISM patients. **C**, Skewing of $CD8^+$ T cells from naive toward central memory phenotype was observed. **D**, A similar trend was seen for $CD4^+$ T cells, although not reaching statistical significance. CD45RA expression on T cells was not different from controls. $*P < .05$, $**P < .01$.

to additional literature study (Table III). According to IPA, the proteins in cluster 1 were related to activation of T cells, dendritic cells, and mast cells, while the proteins in the other 4 clusters were largely related to cell proliferation, cell survival, and apoptosis. However, clusters 2 to 5 also contained several proteins that are involved in type I/II interferon, TNF-α, or IL-18 signaling cascades. Furthermore, IL-6 and oncostatin-M were increased in subgroup A (although assigned to different clusters). Both these cytokines belong to the same cytokine family (the IL-6 family), and elevated serum levels have previously been demonstrated in mastocytosis.[19,20]

DISCUSSION

This exploratory study identified signs of broad immune activation in at least a subset of ISM patients. First, decreased proportions of pDC and monocytes and increased T_H2 cells were observed in peripheral blood of 18 ISM patients compared to healthy controls (cohort 1). A second cohort of 20 ISM patients subsequently showed considerable heterogeneity in the immune proteome; we discerned 2 subgroups. Eight ISM patients within cohort 2 displayed a proinflammatory serum protein signature characterized by markers of T-cell, dendritic cell, and monocyte/macrophage activation and cytokines such as IL-6, TNF-α, and type I/II interferons. The findings in these 2 cohorts thus complement each other and indicate immune activation in systemic mastocytosis that extends beyond mast cells alone, involving several other cellular elements.

Mast cells can influence the immune system through various routes, mostly as a result of their residence in connective tissues where they can communicate with neighboring cells.[21] The decreased numbers of pDC and monocytes as found in peripheral

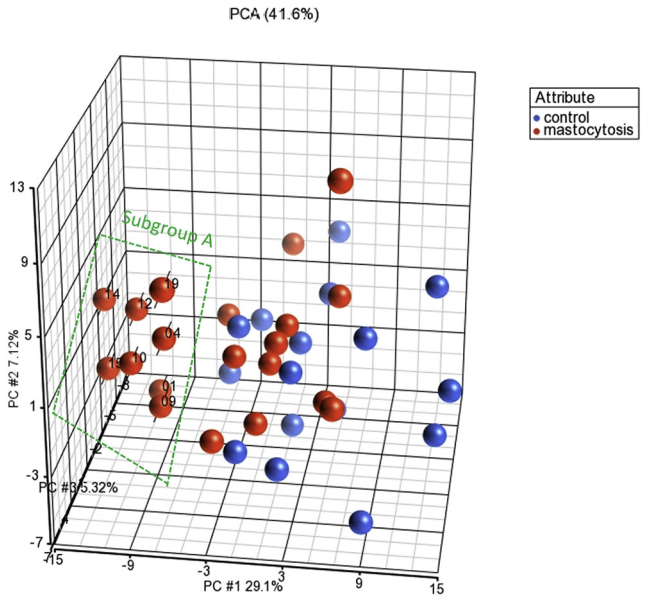

FIG 3. PCA of 20 ISM patients compared to 20 healthy controls (cohort 2). The immune proteome of ISM patients is heterogeneic across cases, and 2 subgroups could be identified. All 8 patients of subgroup A are situated separately from the rest of the patients (subgroup B, n = 12) and controls. Of note, this division was identical to the subgroups as discerned by hierarchical clustering (Fig 4). The subject numbers of subgroup A are indicated in the PCA plot for comparison to Fig 4.

blood from ISM patients might reflect increased recruitment to affected tissues such as the skin, bone marrow, and lymphatic system, driven by the neoplastic mast cells present there. Monocytes undergo drastic phenotypical changes when they enter the tissue and evolve into macrophages, which are key producers of TNF-α and IFN-γ. Much is still unknown on the interaction between mast cells and monocytes/macrophages. Yet the mast cell line HMC1 produces high levels of monocyte chemoattractant protein 1 (MCP-1), an important chemokine for monocytes.[22] Accordingly, *in vitro*–activated mast cells were found to recruit monocytes, then subsequently stimulate them to produce TNF-α.[23] MCP-1 levels were increased in subgroup A of cohort 2 (fold change of 1.29 compared to healthy controls), without reaching statistical significance. The results from both ISM cohorts thus indicate monocyte activation and potential migration into tissues. Other important monocyte chemokines such as C-C motif chemokine ligand (CCL) 5 were not included in the proteomics panels used in our study. We also found that pDC were less abundant in peripheral blood of ISM patients than controls. The pDC is able to produce high levels of type I interferon, as well as IL-6 and TNF-α, which were all upregulated in subgroup A of cohort 2. Interestingly, the mean serum level of CCL19, an important chemokine for pDC, was also significantly elevated in subgroup A. CCL19 is normally expressed in lymphoid tissue but is also found in inflamed skin, where its levels correlate with the presence of dendritic cell/T-cell clusters.[24] Furthermore, the selective influx of pDC and $CD8^+$ dendritic cells in lymphoid tissue is mast cell dependent, with histamine and IL-6 as indispensable mediators.[25] These findings again suggest increased recruitment of inflammatory cells, in particular pDC, to affected tissues, subsequently facilitating T-cell activation.[26]

The skewing from naive to central memory T cells in cohort 1 and the increased serum levels of CD5, CD6, and CD40 observed in subgroup A of cohort 2 support T-cell activation in at least a subset of ISM patients. Furthermore, supernatants from activated mast cells skew dendritic cells toward a T_H2-promoting type,[27] which matches the increased numbers of T_H2 cells we observed. Mast cells can also activate T cells directly through cellular interactions as well as soluble mediators, most importantly IL-6 and TNF-α.[28] Moreover, T-cell–derived IFN-γ increases mast cell–induced T-cell stimulation[29] and suppresses apoptosis of mast cells.[30] In subgroup A of the proteomics cohort, various IFN-γ–related proteins were indeed found to be upregulated (Table E5). Collectively, these data suggest activation of pDC, monocytes, and T cells in ISM, potentially leading to a self-reinforcing cycle of activation induced by proinflammatory signals from neoplastic mast cells, in at least a subset of ISM patients (Fig 5). To our knowledge, infiltration of monocytes/macrophages, pDC, or T cells in lesional skin of mastocytosis patients has not been studied yet.

As mentioned previously, 2 clear subgroups could be distinguished on the basis of the serum proteome in cohort 2. Subgroup A had a proinflammatory protein signature, characterized by upregulation of TLR4–, IFN-γ–, and TNF-α–related pathways. Hierarchical clustering of the 62 significantly upregulated proteins revealed many other proteins involved in cell proliferation and survival. Interestingly, the levels of IL-6 and oncostatin-M were also increased. Both these proinflammatory cytokines are produced by neoplastic mast cells carrying the KIT D816V mutation.[19,20] Elevated serum cytokine levels have been previously described to correlate with the presence of fatigue[6] and constitutional symptoms[31] in systemic mastocytosis, but not to mast cell mediator–related symptoms.[31] This is similar to our findings. It thus appears that inflammatory signaling pathways contribute to constitutional symptoms, but that they are less involved in classical symptoms, such as itch and anaphylaxis. It remains unclear where these inflammatory processes take place. We hypothesize that skin is the main site of interaction of pDC, macrophages, and T cells with mast cells. However, not all patients in ISM subgroup A had skin involvement. Neoplastic mast cells in bone marrow and lymphoid tissue may therefore also be involved in driving inflammation, both locally and systemically.

Next to proinflammatory proteins, several substances related to mast cell degranulation were present in the 62 significantly upregulated proteins of cohort 2. Interestingly, Gulen et al[32] recently published serum proteomics data of 19 patients with mastocytosis, focusing on anaphylaxis rather than inflammation. They found that although the mean Allergin-1 and pappalysin-1 levels were increased in mastocytosis compared to controls, the levels of both substances were significantly lower in patients with anaphylaxis.[32] In our cohort 2, Allergin-1 was upregulated in 12 of 20 mastocytosis subjects ($P = .001$ for comparison to controls). However, we found no significant correlation between Allergin-1 levels and anaphylaxis. This conflicting result from Gulen et al again demonstrates the heterogeneity within ISM and the need for larger cohorts to confirm results.

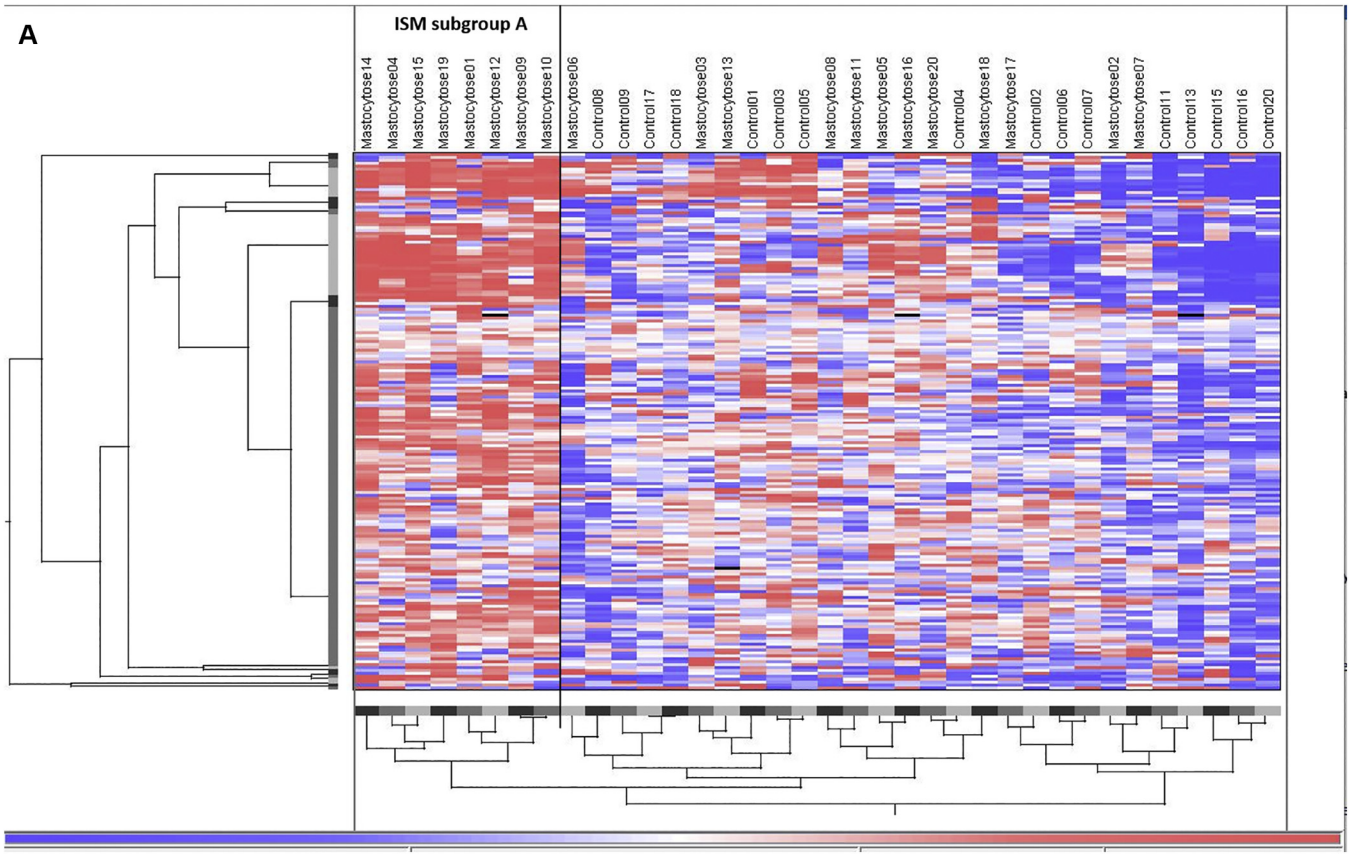

FIG 4. Hierarchical clustering of all inflammation-related proteins as measured by proteomics (cohort 2). **A**, Nonsupervised hierarchical clustering of all 169 analyzed unique proteins revealed 2 distinct subgroups within the mastocytosis cohort. Subgroup A had a clearly different expression profile compared to controls, while 0 proteins were significantly different from controls in subgroup B (n = 12), as can be seen in the clustering plot in which the patients from subgroup B are mixed among controls. **B**, When compared to the healthy controls (n = 20), 62 proteins were significantly overexpressed in subgroup A (n = 8). Scale bar: *red,* increased expression compared to the geometric mean; *blue,* decreased expression compared to the geometric mean. Color intensity correlates with degree of change.

Measurement of derivatives of inflammatory activity in peripheral blood has several caveats. First, some proteins are metabolized quickly or are protein bound, which might hamper detection. Second, mast cells reside in connective tissue and mucosa, and therefore their mediators can be expected at higher levels in affected tissues than in blood. This also accounts for peripheral blood leukocytes, where a decrease might indicate recruitment into another tissue compartment. In systemic mastocytosis, neoplastic mast cells can mostly be found in bone marrow and skin, where they might change the local environment, attracting and activating other leukocytes. Studies examining paired blood and tissue samples in mastocytosis have not yet been published. Another limitation of our study is the relatively small patient cohorts. Although inherent to rare diseases, extrapolation of the results to requires some caution and warrants further studies. Last, the cellular and proteome analyses were conducted in different cohorts. However, the clinical characteristics of both cohorts were comparable. Moreover, the observed alterations in peripheral blood leukocyte subsets match the serum proteome in a pathophysiologic sense, which can also be considered a strength of this study.

In conclusion, this exploratory study revealed decreased proportions of pDC and monocytes and increased T_H2 numbers in peripheral blood of ISM patients. In a subsequent cohort, an inflammatory serum proteome characterized by upregulation of proteins associated with TLR4, IL-6, TNF-α, and interferon type I/II pathways was seen in a subset of ISM patients. This substantiates the hypothesis that several immune components other than mast cells are activated in at least a subset of ISM patients. We propose a pathophysiologic model in which neoplastic mast cells stimulate recruitment and activation of pDC, monocytes, and T cells in the skin or other affected tissues. The subsequent local interplay between these cells creates a vicious cycle leading to the production of proinflammatory cytokines, in particular IL-6, type I/II interferons, and TNF-α (Fig 5). Of note, half of the proteomics cohort did not have a proinflammatory proteome signature, suggesting involvement of other mechanisms in those patients. The pathophysiologic mechanisms underlying the differences between the subgroups remain to be elucidated. Our findings emphasize the importance of considering other immune components in the pathogenesis of mastocytosis and provides grounds for further research.

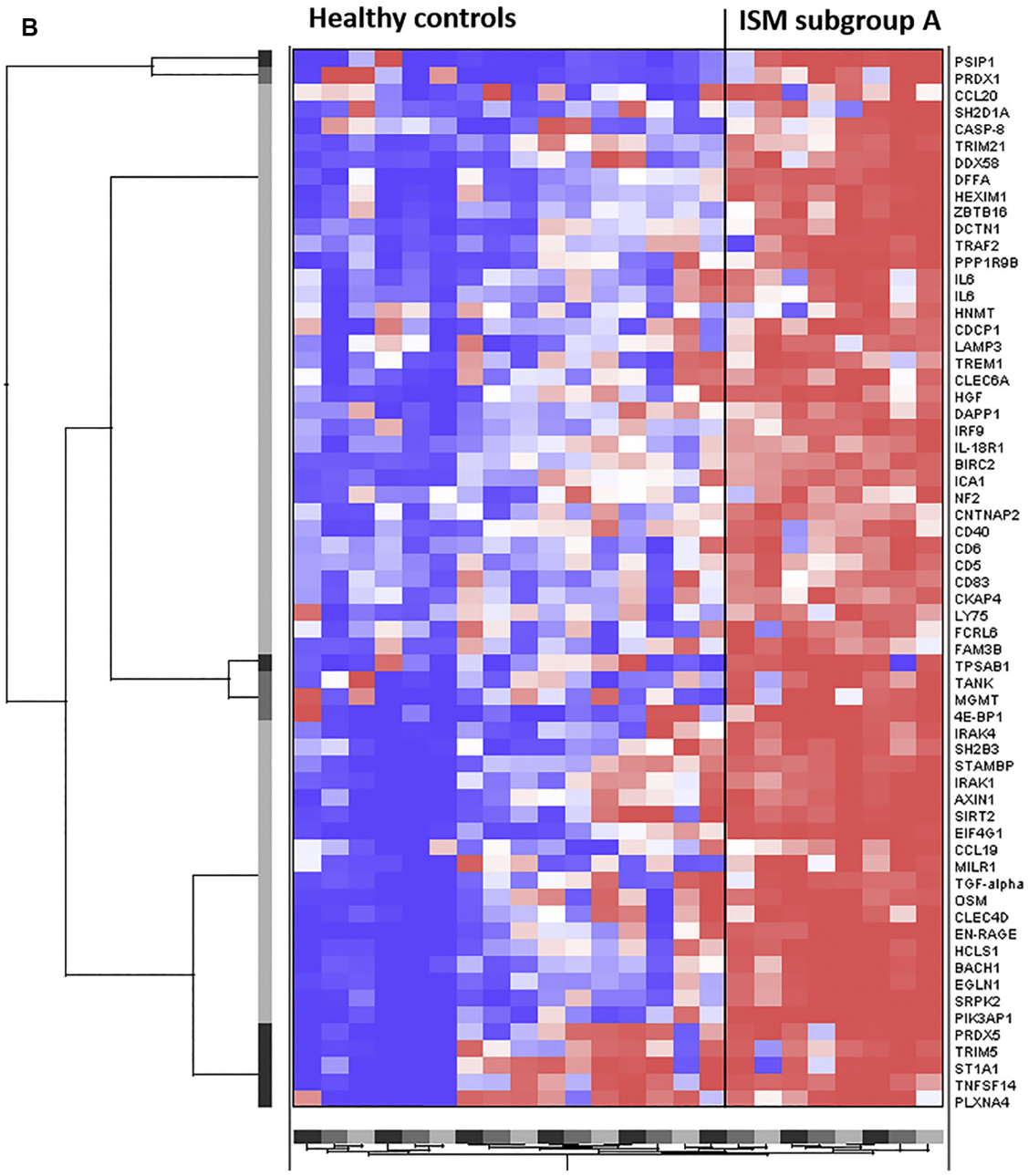

FIG 4. Continued.

TABLE II. Baseline characteristics of proteomics cohort 2 (n = 20), grouped by protein expression pattern

Characteristic	Subgroup A (n = 8)	Subgroup B (n = 12)	*P* value*
Age (years), median (IQR)	48 (34-56)	46 (21-63)	.55
Male sex	3 (37.5)	3 (25)	1.00
Serum tryptase (μg/L), median (IQR)	21.5 (14.7-28.5)	18.2 (12.1-49.5)	.91
D816V mutation detected	7 (87.5)	7/10 (70)†	.38
MPCM	5 (62.5)	9 (75)	.55
Itch	3 (37.5)	8 (66.6)	.19
Anaphylaxis	3 (37.5)	1 (8.3)	.11
Atopy	1 (12.5)	2 (16.7)	1.00
Fatigue	6 (75)	4 (33)	.06
Lumbar spine *t* score, median (IQR)	−0.75 (−2.25 to −0.15)	−0.85 (−1.7 to −0.2)	.73
Femur *t* score, median (IQR)	−0.10 (−1.15 to −0.5)	−0.45 (−1.1 to 0.7)	.73

Data are presented as nos. (%) unless otherwise indicated. Atopy indicates the presence of atopic dermatitis, allergic rhinoconjunctivitis, asthma, or specific IgE against inhalational allergens. The *t* score was measured by bone densitometry.
IQR, Interquartile range.
*Mann-Whitney *U* test for continuous variables and Fisher exact test for dichotomous variables.
†KIT D816V mutation status was unknown in 2 patients.

TABLE III. Clusters of significantly upregulated proteins in subgroup A of proteomics cohort 2 (n = 8)

Protein cluster	Common function*	Name	Fold change	Common function
Cluster 1	Cellular homeostasis	LAMP3	1.793	DC activation
	T-cell activation	CNTNAP2	1.648	Neurologic metabolism
		CCL20	1.754	Lymphocyte and DC chemotaxis, mast cell activation
		CD40	1.519	T-cell activation
		CD5	1.649	T-cell activation
		DDX58	2.024	Type I interferon pathway, T-cell activation
		CD6	1.859	T-cell activation
		CD83	1.539	DC activation
Cluster 2	Cancer	AXIN1	2.358	Cell proliferation
	Apoptosis	SIRT2	2.185	Cell proliferation
		ST1A1	1.671	Neurologic metabolism
		TRIM21	1.967	Type I interferon pathway
		TRIM5	2.133	Type I interferon pathway
		EIF4G1	3.491	Cell proliferation
		STAMBP	1.967	Cell proliferation
		HEXIM1	2.524	Cell proliferation
		DCTN1	2.228	Bone homeostasis
		ZBTB16	2.355	Cell proliferation
		IRAK1	1.997	IL-18 and NF-κB pathway
		CCL19	2.482	T-cell and DC chemotaxis
		BIRC2	1.692	TNF pathway
		CASP8	1.872	IL-18 and IFN-γ pathway
		PSIP1	2.616	Cell proliferation
		MILR1	2.356	Mast cell activation
		PRDX5	2.376	Hypoxic stress
		LY75	1.597	DC activation
Cluster 3	Cancer	ICA1	1.638	Function unclear
	Melanoma	IRF9	1.817	IFN-γ pathway
	Necrosis	HNMT	1.814	Mast cell activation
		SRPK2	4.298	Cell proliferation
		EGLN1	3.493	Erythrocyte proliferation
		EN-RAGE	3.311	IL-6 pathway, general inflammation
		PPP1R9B	2.364	Cell proliferation
		BACH1	2.967	Cell proliferation
		HCLS1	3.493	Lymphocyte activation, mast cell activation
		PIK3AP1	4.333	Lymphocyte activation
		TANK	1.963	T-cell activation
		CDCP1	1.958	Cell migration, DC activation
		CKAP4	1.608	Cell migration
		PRDX1	4.023	Hypoxic stress
		IL-18R1	1.642	IL-18, NF-κB, and IFN-γ pathways
		MGMT	2.553	Cell proliferation
Cluster 4	Cell proliferation	TPSAB1	2.405	Mast cell activation, activation of innate immunity, wound healing
	Apoptosis	HGF	1.845	IFN-γ and TNF pathways
		CLEC4D	2.052	T-cell activation, mast cell activation
		TREM1	1.555	Lymphocyte and granulocyte activation, hypoxic stress
		TRAF2	1.977	TNF and NF-κB pathways
		NF2	1.505	Cell proliferation
		4E-BP1	2.638	Cell proliferation
		OSM	2.233	T-cell activation, mast cell activation
		TGF-alpha	1.989	Cell proliferation
Cluster 5	Melanoma	SH2B3	2.155	Cell proliferation
	Proliferation of lymphocytes	IRAK4	2.330	T-cell activation, IL-18 and NF-κB pathway
		IL-6	2.055	Innate immune activation, mast cell activation
		DAPP1	1.562	Mast cell activation
		CLEC6A	1.751	Mast cell activation
		PLXNA4	1.651	NF-κB pathway
		TNFSF14	1.723	T-cell activation, cell survival
Not in any particular cluster		SH2D1A	2.025	T-cell activation
		DFFA	2.572	Cell survival
		FCRL6	1.727	T-cell activation
		FAM3B	1.714	Cell survival

DC, Dendritic cell; *NF-κB,* nuclear factor kappa–light-chain enhancer of activated B cells.
*Analyzed by IPA.

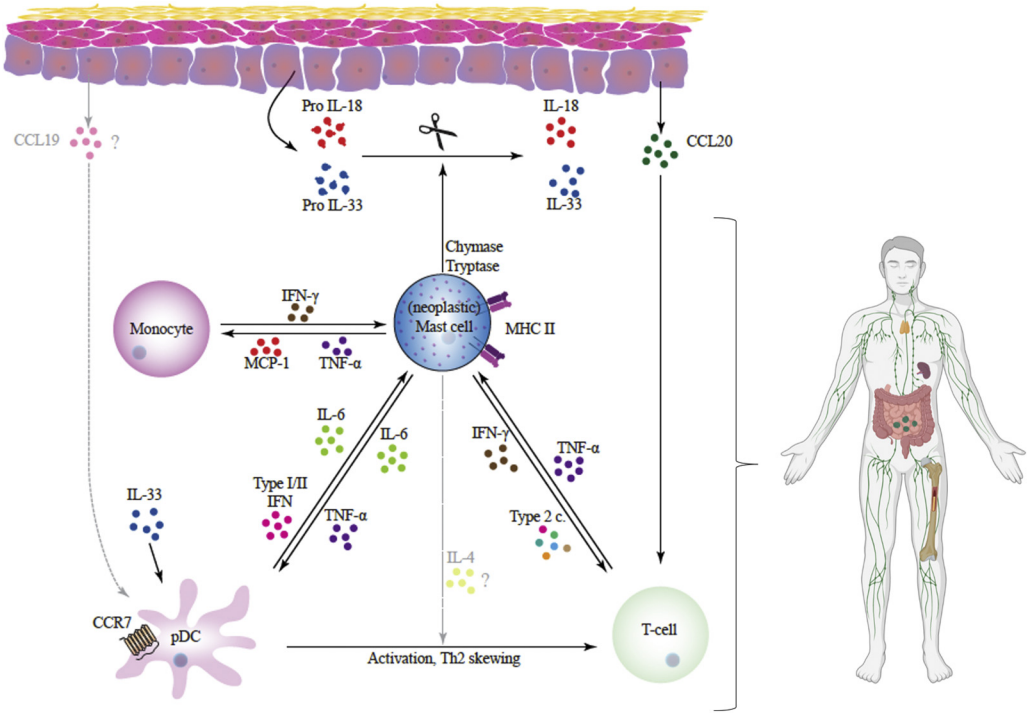

FIG 5. Hypothetical model of general inflammation induced by neoplastic mast cells. Skin was taken as an example, but similar mechanisms might take place in other organs such as lymphoid organs, bone marrow, or gut. Neoplastic mast cells (MC) produce proteases that affect the skin environment and activate keratinocytes to produce CCL19 to recruit pDC and naive T cells from circulation. MC also produce high levels of MCP-1, attracting monocytes into the skin. Subsequently, MC can stimulate activation of pDC, monocytes, and T cells by the production of soluble mediators (most importantly IL-6, TNF-α, and histamine) by cleaving premature forms of IL-18 and IL-33, as well as by direct cell-to-cell contact. Furthermore, MC induce T_H2 polarization by pDC and potentially also directly via the production of IL-4. As a result, a vicious cycle of proinflammatory signals arises between all 4 involved cell types. This leads to decreased numbers of circulating pDC and monocytes and skewing of the T-helper cell compartment toward T_H2, as well as a proinflammatory serum proteome. A similar process to that described above might take place in other shelters of neoplastic MC such as bone marrow.

Clinical implications: Characterization of inflammatory mechanisms in ISM might uncover additional targets for treatment, in particular for constitutional symptoms such as fatigue.

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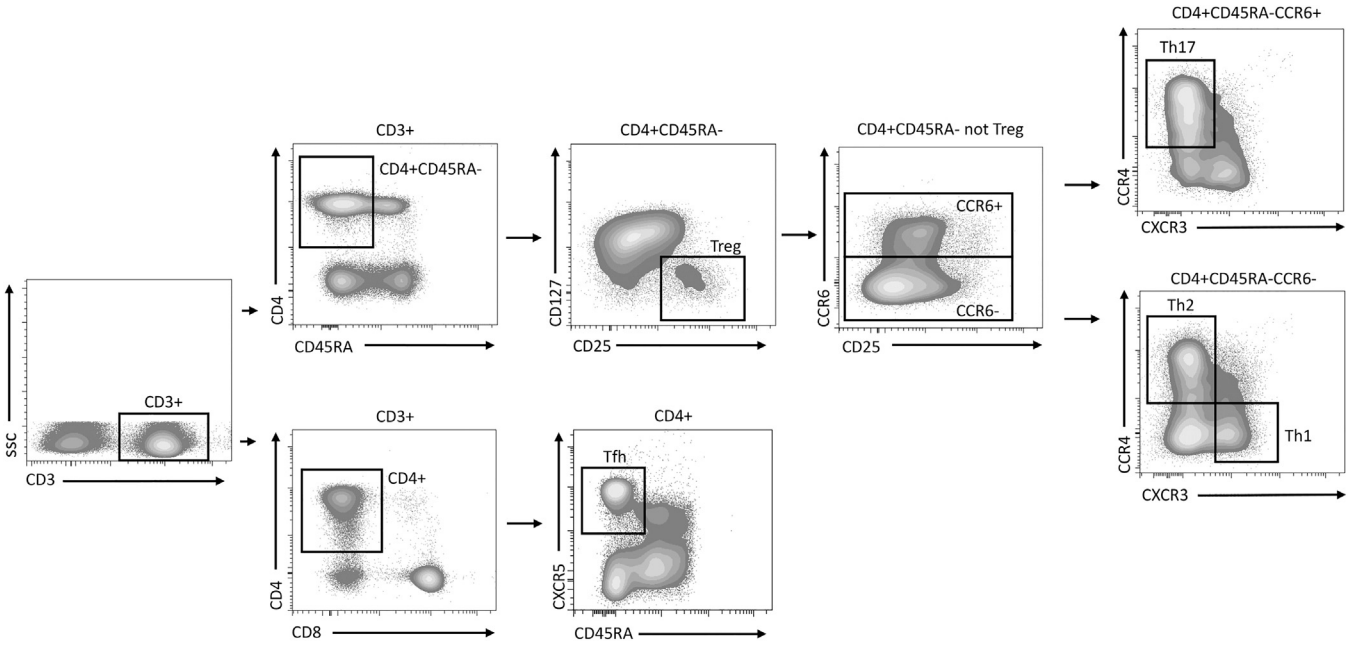

FIG E1. Gating strategy of T-helper cell subsets for cohort 1.

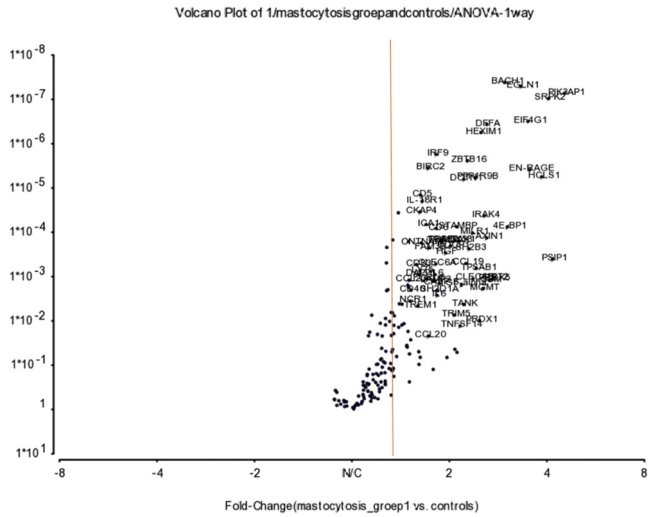

FIG E2. Volcano plot of 62 significant proteins of subgroup A of cohort 2. Volcano plot of all significant proteins in subgroup A showed that no proteins were relevantly downregulated in ISM patients compared to controls (statistically analyzed with ANOVA).

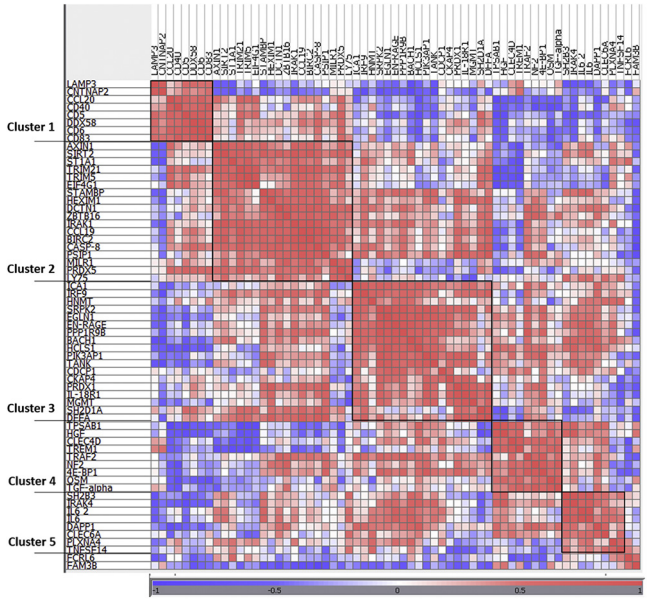

FIG E3. Hierarchical clustering of 62 significant proteins of subgroup A of cohort 2. Hierarchical clustering of the 62 proteins that were overexpressed in ISM subgroup A reveals 5 clusters that cosegregate with each other. Scale bar: *red* indicates increased expression compared to the geometric mean; *blue,* decreased expression compared to the geometric mean. Color intensity correlates with degree of change.

TABLE E1. Antibodies used for flow cytometry

Subsets	Antibody	Conjugate	Clone	Supplier
T-effector cell subsets	CD3	BV711	UCHT1	BD Biosciences, San Jose, Calif
	CD4	BV510	OKT4	BioLegend, San Diego, Calif
	CD8	APC-H7	SK1	BD Biosciences
	CD27	BV421	M-T271	BD Biosciences
	CD28	PerCP-Cy5.5	CD28.2	BioLegend
	CD45RA	BV605	HI100	BioLegend
	CD45RO	FITC	UCHL1	EXBIO, Prague, Czech Republic
	CCR7	PE	REA108	Miltenyi Biotec, Bergisch Gladbach, Germany
	CXCR5	APC	RF8B2	BD Biosciences
T-helper cell subsets	CD3	BV711	UCHT1	BD Biosciences
	CD4	BV510	OKT4	BioLegend
	CD8	APC-H7	SK1	BD Biosciences
	CD25	BV421	BC96	BioLegend
	CD45RA	BV605	HI100	BioLegend
	CD127	APC	A019D5	BioLegend
	CCR4	PE-Cy7	L291H4	BioLegend
	CCR6	PerCP-Cy5.5	G034E3	BioLegend
	CCR10	PE	314305	R&D Systems, Minneapolis, Minn
	CXCR3	FITC	G025H7	BioLegend
Leukocyte subsets	CD15	PE-CF594	W6D6	BD Biosciences
	CD45	OC515	GA90	Cytognos, Santa Marta de Tormes, Spain
	CD25	BV421	BC96	BioLegend
	CD38	APC-H7	HB7	BD Biosciences
	CD117	APC	104D2	BD Biosciences
	CD123	PE-Cy7	6H6	eBioscience, San Diego, Calif
	CD203c	PE	97A6	Beckman Coulter, Fullerton, Calif
	HLA-DR	BV605	L243	BioLegend
	IgE	FITC	Goat anti-human	Invitrogen; Thermo Fisher Scientific, Waltham, Mass

APC, Allophycocyanin; *FITC,* fluorescein isothiocyanate; *PE,* phycoerythrin.

TABLE E2. Immunophenotypic definitions of T-cell subsets and leukocytes

Characteristic	Immunophenotype
$CD3^+$ T-cell subset	
Total $CD8^+$ T cell	$CD3^+CD8^+$
$CD8^+$ naive T cell	$CD3^+CD8^+CCR7^+CD45RO^-$
$CD8^+$ central memory T cell (Tcm)	$CD3^+CD8^+CCR7^+CD45RO^+$
$CD8^+$ effector memory RO T cell (TemRO)	$CD3^+CD8^+CCR7^-CD45RO^+$
$CD8^+$ effector memory RA T cell (TemRA)	$CD3^+CD8^+CCR7^-CD45RO^-$
Total $CD4^+$ T cell	$CD3^+CD4^+$
$CD4^+$ naive T cell	$CD3^+CD4^+CCR7^+CD45RO^-$
$CD4^+$ Tcm	$CD3^+CD4^+CCR7^+CD45RO^+$
$CD4^+$ TemRO	$CD3^+CD4^+CCR7^-CD45RO^+$
$CD4^+$ TemRA	$CD3^+CD4^+CCR7^-CD45RO^-$
T_H1	$CD3^+CD4^+CD45RA^-CCR6^-CCR4^-CXCR3^+$
T_H2	$CD3^+CD4^+CD45RA^-CCR6^-CCR4^+CXCR3^-$
$CD45^+$ leukocyte subsets	
Granulocyte	$CD45^+SSC^{high}$
Neutrophil	$CD45^+SSC^{high}CD15^+$
Eosinophil	$CD45^+SSC^{high}CD15^-$
Basophil	$CD45^{dim}SSC^{low}HLADR^-CD123^{+-}$
Monocyte	$CD45^+SSC^{low}$
pDC	$CD45^{dim}SSC^{low}HLADR^+CD123^{+-}$

TABLE E3. Proteins included in both proteomics panels (Olink, Uppsala, Sweden)

Panel	Protein name	Abbreviation
Inflammation panel (n = 89)*		
1	Adenosine deaminase	ADA
2	Artemin	ARTN
3	Axin-1	AXIN1
4	Beta-nerve growth factor	Beta-NGF
5	C-C motif chemokine 19	CCL19
6	C-C motif chemokine 20	CCL20
7	C-C motif chemokine 23	CCL23
8	C-C motif chemokine 25	CCL25
9	C-C motif chemokine 28	CCL28
10	C-C motif chemokine 3	CCL3
11	C-C motif chemokine 4	CCL4
12	C-C motif chemokine 11	CCL11 (eotaxin)
13	C-X-C motif chemokine 1	CXCL1
14	C-X-C motif chemokine 10	CXCL10
15	C-X-C motif chemokine 11	CXCL11
16	C-X-C motif chemokine 5	CXCL5
17	C-X-C motif chemokine 6	CXCL6
18	C-X-C motif chemokine 9	CXCL9
19	Caspase-8	CASP-8
20	CD40L receptor	CD40
21	CUB domain–containing protein 1	CDCP1
22	Cystatin D	CST5
23	Delta and Notch-like epidermal growth factor–related receptor	DNER
24	Eukaryotic translation initiation factor 4E–binding protein 1	4E-BP1
25	Fibroblast growth factor 19	FGF-19
26	Fibroblast growth factor 21	FGF-21
27	Fibroblast growth factor 23	FGF-23
28	Fibroblast growth factor 5	FGF-5
29	Fms-related tyrosine kinase 3 ligand	Flt3L
30	Fractalkine	CXCL3CL1
31	Glial cell line–derived neurotrophic factor	GDNF
32	Hepatocyte growth factor	HGF
33	Interferon gamma	IFN-gamma
34	Interleukin-1 alpha	IL-1 alpha
35	Interleukin-10	IL-10
36	Interleukin-10 receptor subunit alpha	IL-10RA
37	Interleukin-10 receptor subunit beta	IL-10RB
38	Interleukin-13	IL-13
39	Interleukin-15 receptor subunit alpha	IL-15RA
40	Interleukin-17A	IL-17A
41	Interleukin-17C	IL-17C
42	Interleukin-18	IL-18
43	Interleukin-18 receptor 1	IL-18R1
44	Interleukin-2	IL-2
45	Interleukin-2 receptor subunit beta	IL-2RB
46	Interleukin-20	IL-20
47	Interleukin-20 receptor unit alpha	IL-20RA
48	Interleukin-22 receptor unit alpha-1	IL-22RA1
49	Interleukin-24	IL-24
50	Interleukin-33	IL-33
51	Interleukin-4	IL-4
52	Interleukin-5	IL-5
53	Interleukin-6	IL-6
54	Interleukin-7	IL-7
55	Interleukin-8	IL-8
56	Latency-associated peptide transforming growth factor beta-1	—
57	Leukemia inhibitory factor	LIF
58	Leukemia inhibitory factor receptor	LIF-R
59	Macrophage colony-stimulating factor 1	CSF-1
60	Matrix metalloproteinase-1	MMP-1
61	Matrix metalloproteinase-10	MMP-10

(Continued)

TABLE E3. *(Continued)*

Panel	Protein name	Abbreviation
62	Monocyte chemotactic protein-1	MCP-1
63	Monocyte chemotactic protein-2	MCP-2
64	Monocyte chemotactic protein-3	MCP-3
65	Monocyte chemotactic protein-4	MCP-4
66	Natural killer cell receptor 2B4	CD244
67	Neutrophin-3	NT-3
68	Neurturin	NRTN
69	Oncostatin-M	OSM
70	Osteoprotegerin	OPG
71	Programmed cell death 1 ligand	PD-L1
72	Protein S100-A12	EN-RAGE
73	Signaling lymphocyte activation molecule	SLAMF1
74	SIR2-like protein 2	SIRT2
75	STAM-binding protein	STAMBP
76	Stem cell factor	SCF
77	Sulfotransferase 1A1	ST1A1
78	T-cell surface glycoprotein CD8 alpha chain	CD8A
79	Thymic stromal lymphoprotein	TSLP
80	TNF-beta	TNFB
81	TNF-related activation-induced cytokine	TRANCE
82	TNF-related apoptosis-inducing ligand	TRAIL
83	Transforming growth factor alpha	TGF-alpha
84	Tumor necrosis factor	TNFB
85	Tumor necrosis factor superfamily, member 12	TNFSF12
86	Tumor necrosis factor superfamily, member 14	TNFSF14 (TWEAK)
87	Tumor necrosis factor superfamily, member 9	TNFSF9
88	Urokinase-type plasminogen activator	uPA
89	Vascular endothelial growth factor A	VEGF-A
Immune response panel (n = 84)*		
1	Allergin-1	MILR1
2	Amphiregulin-1	AREG
3	Aryl hydrocarbon receptor nuclear translocator	ARNT
4	Baculoviral IAP repeat-containing protein 2	BIRC2
58	Beta-galactosidase	GLB1
6	Butyrophilin subfamily 3 member A2	BTN3A2
7	C-C motif ligand 11	CCL11 (eotaxin)
8	C-type lectin domain family 4 member A	CLEC4A
9	C-type lectin domain family 4 member C	CLEC4C
10	C-type lectin domain family 4 member D	CLEC4D
11	C-type lectin domain family 4 member G	CLEC4G
12	C-type lectin domain family 6 member A	CLEC6A
13	C-type lectin domain family 7 member A	CLEC7A
14	CD83 antigen	CD83
15	Contact-associated protein-like 2	CNTNAP2
16	Corneodesmosin	CDSN
17	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1
18	Coxsackievirus and adenovirus receptor	CXADR
19	C-X-C motif chemokine 12	CXCL12
20	Cytoskeleton-associated protein 4	CKAP4
21	Diacylglycerol kinase zeta	DGKZ
22	Discoidin, CUB, and LCCL domain-containing protein 2	DCBLD2
23	DNA fragmentation factor subunit alpha	DFFA
24	Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide	DAPP1
25	Dynactin subunit 1	DCTN1
26	E3 ubiquitin-protein ligase TRIM21	TRIM21
27	Egl nine homolog 1	EGLN1
28	Eukaryotic translation initiation factor 4 gamma 1	EIF4G1
29	Eukaryotic translation initiation factor 5A	EIF5A
30	Hematopoietic lineage cell-specific protein	HCLS1
31	Histamine N-methyltransferase	HNMT
32	Importin subunit alpha-5	KPNA1
33	Inactive dipeptidyl peptidase 10	DPP10

(Continued)

TABLE E3. *(Continued)*

Panel	Protein name	Abbreviation
34	Integral membrane protein 2A	ITM2A
35	Integrin alpha-11	ITGA11
36	Integrin alpha-6	ITGA6
37	Integrin beta-6	ITGB6
38	Interferon lambda receptor 1	IFNLR1
39	Interferon regulatory factor 9	IRF9
40	Interleukin-1 receptor-associated kinase 1	IRAK1
41	Interleukin-1 receptor-associated kinase 4	IRAK4
42	Interleukin-10	IL-10
43	Interleukin-12 subunit beta-1	IL-12RB1
44	Interleukin-5	IL-5
45	Interleukin-6	IL-6
46	Islet cell autoantigen 1	ICA1
47	Keratin type 1 cytoskeletal 19	KRT19
48	Leukocyte immunoglobulin-like receptor subfamily B member 4	LILRB4
49	Lymphocyte activation gene 3 protein	LAG3
50	Lymphocyte antigen 75	LY75
51	Lysosome-associated membrane glycoprotein 3	LAMP3
52	Mannan-binding lectin serine protease 1	MASP1
53	Merlin	NF-2
54	Methylated-DNA-protein-cysteine methyltransferase	MGMT
55	Natural cytotoxicity triggering receptor 1	NCR1
56	Natural killers cell antigen CD94	CD94 (KLRD1)
57	Nuclear factor of activated TR cells, cytoplasmic 3	NFATC3
58	Parathyroid hormone/parathyroid hormone-related peptide receptor	PTH1R
59	PC4 and SFRS1-interacting protein	PSIP1
60	Peroxiredoxin-1	PRDX1
61	Peroxiredoxin-5, mitochondrial	PRDX5
62	Phosphoinositide 3-kinase adapter protein-1	PIK3AP1
63	Plexin-A4	PLXNA4
64	Polypeptide N-acetylgalactosaminyltransferase 3	GALNT3
65	Probable ATP-dependent RNA helicase DDX58	DDX58
66	Protein FAM38	FAM38
67	Protein HEXIM1	HEXIM1
68	Protein kinase C theta type	PRKCQ
69	Protein sprouty homolog 2	SPRY2
70	Protein-arginine deiminase type-2	PADI2
71	SH2 domain-containing protein 1A	SH2D1A
72	SH2B adapter protein 3	SH2B3
73	Stanniocalcin-1	STC1
74	T-cell specific surface glycoprotein CD28	CD28
75	Thioredoxin-dependent peroxide reductase, mitochondrial	PRDX3
76	TNF receptor associated factor 2	TRAF2
77	TRAF family member–associated NF-kappa-B activator	TANK
78	Transcription factor AP-1	JUN
79	Transcription regulator protein BACH1	BACH1
80	Triggering receptor expressed on myeloid cells 1	TREM1
81	Tripartite motif–containing protein 5	TRIM5
82	Tryptase alpha/beta-1	TPSAB1
83	Tumor necrosis factor superfamily member EDAR	EDAR
84	Zinc finger and BTB domain–containing protein 16	ZBTB16

*A total of 169 unique proteins were measured as a result of some overlap in the 2 panels (CCL11, IL-10, IL-5, IL-6).

TABLE E4. Detailed patient characteristics

Patient	Cohort and subgroup	Age (years)	Sex	Tryptase (μg/L)	D816V detectable	MPCM	Anaphylaxis	Fatigue	Itch	Diarrhea	Decreased BMD
1	Flow cytometry	43	M	29.6	+	+	−	+	+	+	1
2	Flow cytometry	42	F	16.2	+	+	−	+	+	−	0
3	Flow cytometry	60	M	74.3	+	−	+	−	−	−	1
4	Flow cytometry	50	F	79.2	+	+	+	−	−	−	0
5	Flow cytometry	51	F	11.3	+	+	−	−	+	+	+
6	Flow cytometry	49	F	10.9	+	+	−	−	+	+	+
7	Flow cytometry	51	F	28.2	NA	+	+	+	−	−	1
8	Flow cytometry	67	M	134	−	+	−	−	+	−	0
9	Flow cytometry	66	F	118	NA	+	−	−	−	−	+
10	Flow cytometry	60	F	36.9	+	+	−	−	−	−	0
11	Flow cytometry	48	F	96.3	+	+	−	−	+	−	0
12	Flow cytometry	56	M	13.4	NA	+	−	−	+	−	+
13	Flow cytometry	53	F	71	+	+	−	−	+	−	0
14	Flow cytometry	46	F	28.3	+	+	−	+	−	−	0
15	Flow cytometry	52	F	26.2	NA	−	+	−	−	−	0
16	Flow cytometry	31	F	20.5	−	+	−	−	+	+	+
17	Flow cytometry	83	M	44.2	+	+	+	+	−	−	1
18	Flow cytometry	55	M	25.9	NA	−	+	+	−	−	+
19	Proteomics subgroup A	36	M	20.6	+	−	−	+	−	−	0
20	Proteomics	25	F	4.4	+	+	+	−	−	−	0
21	Subgroup A	49	F	27.7	+	+	−	+	−	+	+
22	Proteomics Subgroup A	47	M	22.3	−	−	−	+	+	−	+
23	Proteomics Subgroup A	59	M	49.1	+	+	+	−	−	+	0
24	Proteomics subgroup A	60	F	15	+	−	+	+	−	−	+
25	Proteomics subgroup A	32	F	14.3	+	+	−	+	+	−	+
26	Proteomics Subgroup A.	53	F	29.3	+	+	−	+	+	+	+
27	Proteomics subgroup B	48	F	57	+	−	−	+	−	−	1
28	Proteomics subgroup B	39	F	14.1	−	−	+	−	+	−	NA
29	Proteomics subgroup B	33	F	9	+	+	−	−	+	+	0
30	Proteomics subgroup B	48	F	22.3	−	−	−	−	+	+	+
31	Proteomics subgroup B	63	F	11	−	+	−	+	−	+	0
32	Proteomics subgroup B	59	M	22.5	+	+	−	−	+	−	+
33	Proteomics subgroup B	39	F	13.1	+	+	−	+	+	−	0
34	Proteomics subgroup B	52	F	8.2	+	+	−	−	−	−	0
35	Proteomics subgroup B	44	M	97	+	+	−	−	+	+	+
36	Proteomics subgroup B	42	M	13.7	NA	+	−	+	+	+	+
37	Proteomics subgroup B	60	F	95.6	NA	+	−	−	+	−	+
38	Proteomics subgroup B	21	F	42.3	+	+	−	−	−	+	+

NA, Not available.

TABLE E5. Top 3 of upstream pathways associated with protein expression patterns in subgroup A of proteomics cohort

Start of pathway	No. of proteins involved	*z* score	*P* value
Toll-like receptor 4	28/62	4.192	6.20×10^{-14}
Tumor necrosis factor alpha	20/62	4.043	1.73×10^{-8}
Interferon-gamma	18/62	3.835	4.95×10^{-8}

Analyzed by IPA.