

Letter to the Editor

Expression of programmed cell death ligand-1 in mastocytosis correlates with disease severity

To the Editor:

Mastocytosis is characterized by clonal expansion of mast cells (MCs) in skin, bone marrow (BM), and other tissues.¹ Most patients carry activating mutations of the *KIT* gene. According to phenotype and extent of MC infiltration, mastocytosis is classified into cutaneous mastocytosis (CM), indolent systemic mastocytosis (ISM), systemic mastocytosis with associated hematological non-MC lineage disease (SM-AHNMD), aggressive systemic mastocytosis (ASM), and mast cell leukemia (MCL). Clinical manifestation is heterogeneous and can include MC mediator symptoms, such as anaphylaxis, diarrhea, and pruritus, and dysfunction of various organ systems, such as cytopenia, hepatosplenomegaly, and weight loss.² In patients with ASM and MCL, the prognosis is poor with a median survival of less than 3 years.

Treatment options in mastocytosis are limited. Patients with non-advanced subtypes are mainly treated by avoidance of MC mediator release and H1 antihistamines. Management of advanced categories comprises IFN- α , cladribine (2-chlorodeoxyadenosine), allogeneic hematopoietic stem cell transplantation, and tyrosine kinase inhibitors targeting *KIT* activation.

Several biomarkers with diagnostic and therapeutic potential have recently been characterized in mastocytosis. Among those, the MC-derived enzyme tryptase is the best-known serum marker, which has been found to closely correlate with the extent of MC infiltration. Other biomarkers include IL-6, IL-31, carboxypeptidase A, neuropeptides, urinary histamine metabolites, and bone cytokines.^{3,4}

Programmed death-1 (PD-1) is a key immune checkpoint receptor expressed on activated T cells, B cells, and cutaneous MCs.^{5,6} By interacting with its ligands, programmed cell death ligand-1 (PD-L1) and programmed cell death ligand-2 (PD-L2), T-cell tolerance protecting tissues from immune-mediated damage is ensured. In humans, PD-L1 is expressed on activated T cells, dendritic cells, and monocytes, whereas expression of PD-L2 is restricted to dendritic cells and monocytes.

In addition, tumor cells in a large variety of solid tumors and hematologic malignancies have been found to express PD-L1. In many tumors, expression levels of PD-L1 correlate with an unfavorable prognosis. In accordance, treatment of these tumors, such as advanced melanoma, lung carcinoma, renal cell carcinoma, and Hodgkin's lymphoma, with antibodies against PD-1 (nivolumab and lambrolizumab) or PD-L1 (BMS-936559) has been demonstrated to be highly effective.^{5,7-9} Immune checkpoint blockade by these antibodies promotes antitumor activity by enhancing immune responses, such as the cytotoxic function of tumor-specific T cells.

To our knowledge, the PD-1–PD-L axis in mastocytosis has not yet been examined. We therefore aimed to investigate whether PD-L1, PD-L2, and PD-1 levels are altered in the serum of patients with mastocytosis and whether these proteins are expressed in mastocytosis infiltrates.

Levels of PD-L1, PD-L2, PD-1, and tryptase were analyzed in the serum of 43 patients with different categories of mastocytosis (adults, $n = 31$; children, $n = 12$), classified according to established World Health Organization's criteria,¹ and 22 healthy

controls (adults, $n = 10$; children, $n = 12$), as detailed in this article's [Methods](#) section in the Online Repository at www.jacionline.org. Detailed clinical characteristics of all patients and controls are listed in [Table E1](#) in this article's Online Repository at www.jacionline.org. Levels of PD-L1, PD-L2, and PD-1 were also measured in cell culture supernatants of different cell lines (see this article's [Methods](#) section in the Online Repository). Furthermore, BM and skin biopsies were stained with antibodies against PD-L1, PD-L2, PD-1, and tryptase by immunofluorescence (see this article's [Methods](#) section in the Online Repository).

Serum levels of PD-L1 were significantly increased in adult patients with mastocytosis compared with adult healthy controls ($P < .0001$; [Fig 1, A](#)). Interestingly, patients with advanced categories, such as SM-AHNMD, ASM, and MCL, exhibited significantly elevated PD-L1 levels compared with those with non-advanced categories, including mastocytosis in the skin (MIS) and ISM ($P = .0022$; [Fig 1, B and C](#)). As anticipated, tryptase levels increased along with disease severity (see [Fig E1](#) in this article's Online Repository at www.jacionline.org), and, consistently, PD-L1 levels were found to also correlate with tryptase levels in adult patients ($P < .0001$; [Fig 1, D](#); see [Fig E2](#) in this article's Online Repository at www.jacionline.org).

To investigate whether MCs are able to produce PD-L1, PD-L2, and PD-1, supernatants of the human MC lines HMC1 and ROSA KIT were investigated ([Fig 1, E](#)). In the 2 HMC1 subclones (HMC1.1 and HMC1.2) and in both ROSA KIT cell lines (ROSA KIT WT and ROSA KIT D816V), we detected significant levels of PD-L1 in supernatants. Of note, production of PD-L1 was more pronounced in HMC1.2 and ROSA KIT D816V cells, which carry the mastocytosis-associated mutation *KIT* D816V, compared with HMC1.1 and ROSA KIT WT cells, which lack this mutation (HMC1, $P = .0084$; ROSA KIT, $P = .0282$; [Fig 1, E](#)). Both cell lines also produced low levels of PD-L2 and PD-1; there was no difference between the 2 HMC1 subclones and the 2 ROSA KIT cell lines (data not shown).

In contrast to PD-L1, we did not observe altered serum levels of PD-L2 in adult patients ([Fig 1, F](#)), but a trend of increased PD-1 levels in patients with advanced mastocytosis categories ([Fig 1, G](#), and [Fig E2](#)).

Unexpectedly, when we compared serum levels of PD-L1, PD-L2, and PD-1 in the control groups of 10 adults and 12 children (see [Fig E3](#) in this article's Online Repository at www.jacionline.org), we found significantly enhanced levels of PD-L1 ([Fig E3, C](#)) and PD-1 ([Fig E3, G](#)) in control children compared to adults. There were no significant differences in PD-L1 and PD-1 levels between pediatric patients with mastocytosis and pediatric controls ([Fig E3, D and H](#)).

We next analyzed BM and skin biopsies of patients with mastocytosis by immunofluorescence using specific antibodies against PD-L1, PD-L2, PD-1, and tryptase ([Fig 2](#)). In both tissues, expression of PD-L1 clearly colocalized with tryptase-positive MC ([Fig 2, A and D](#)). However, confirming our findings on serum levels, we did not observe colocalization of PD-L2–positive cells with tryptase-positive MC, neither in BM ([Fig 2, B](#)) nor in skin tissue ([Fig 2, E](#)). Although tryptase-positive MC in BM failed to express PD-1 ([Fig 2, C](#)), labeling in skin revealed marked expression of PD-1 on MC ([Fig 2, F](#)).

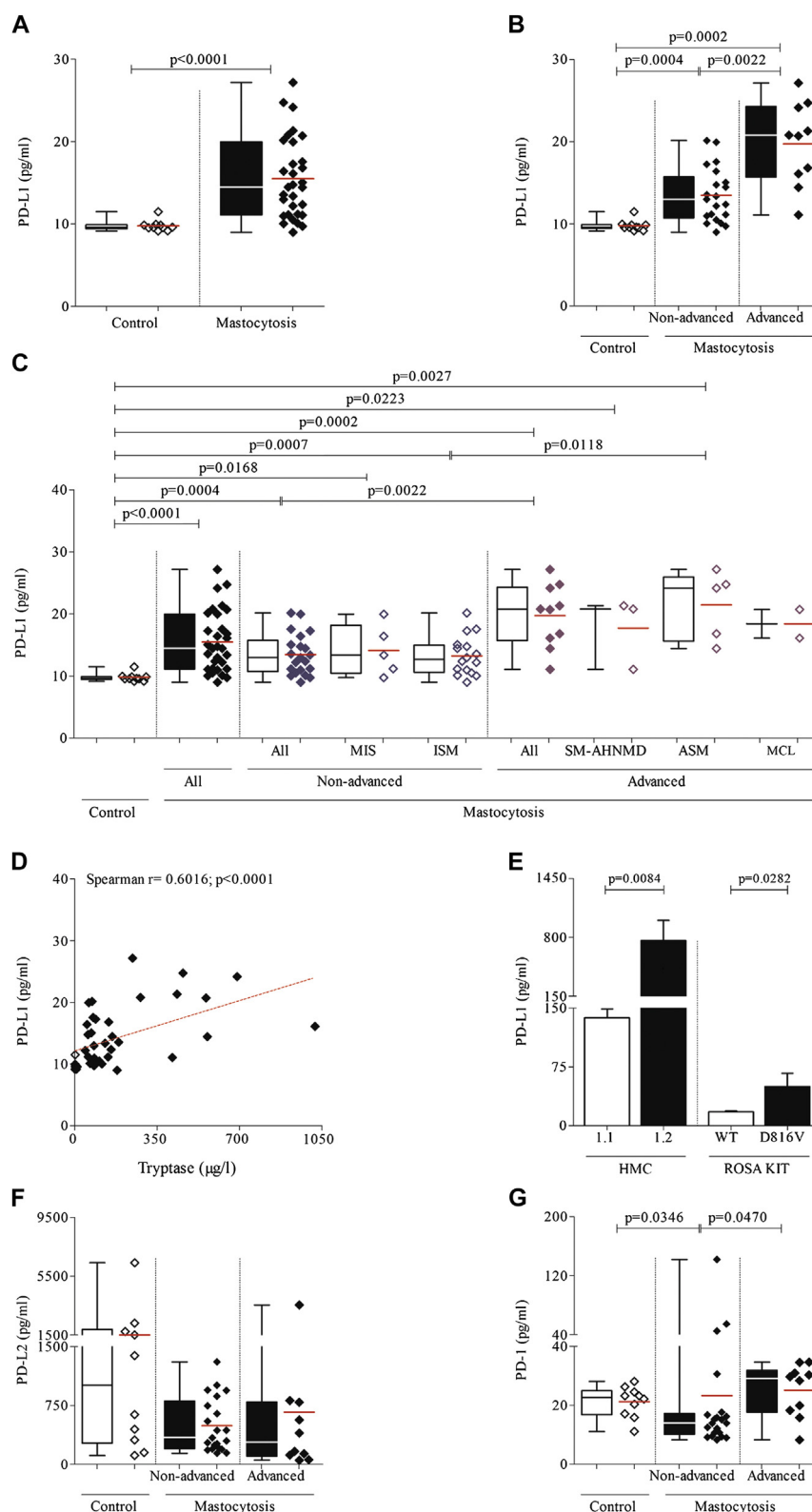


FIG 1. A, PD-L1 serum levels are significantly elevated in adult patients with mastocytosis (Mastocytosis) compared with healthy controls (Control). Data are presented as *box plots* (min to max) with medians (*left*) and scatter dot plots with means (*right*). **B** and **C**, Patients with advanced mastocytosis categories (SM-AHNMD, ASM, and MCL) show increased PD-L1 levels compared to those with non-advanced categories (MIS and ISM). **D**, PD-L1 levels (y-axis) positively correlate with tryptase levels (x-axis) in adult patients with mastocytosis. **E**, PD-L1 levels in supernatants of cultured MCs. PD-L2 (**F**) and PD-1 (**G**) serum levels in adult patients with non-advanced and advanced mastocytosis compared with healthy controls.

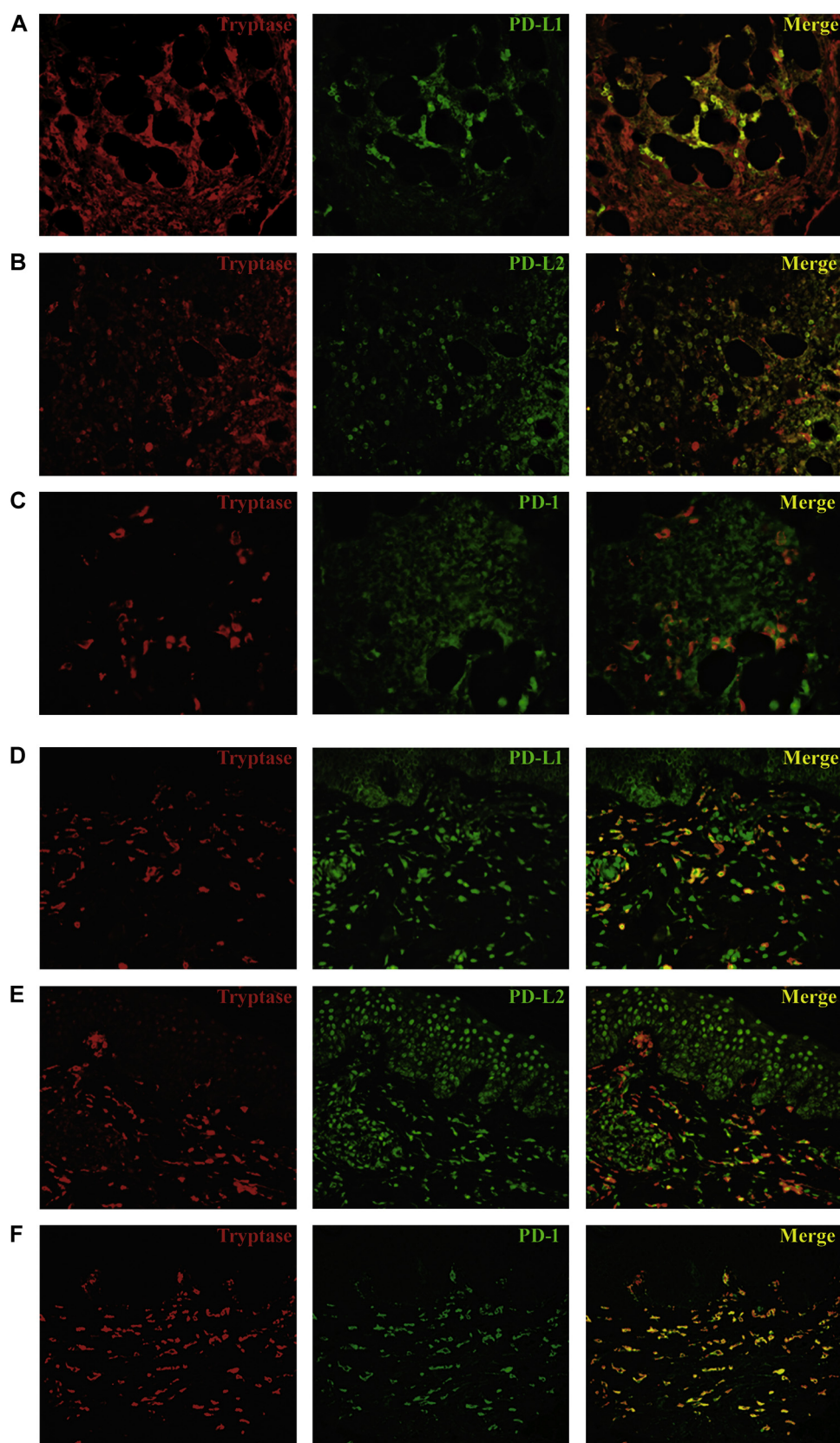


FIG 2. Paraffin-embedded BM (A-C) and skin biopsies (D-F) of patients with systemic mastocytosis analyzed by immunofluorescence. Expression of tryptase is shown in red (left panel), and expression of PD-L1 (Fig 2, A and D), PD-L2 (Fig 2, B and E), and PD-1 (Fig 2, C and F) is shown in green (middle panel). Colocalization is demonstrated by yellow labeling of the cells in merged pictures (right panel).

Taken together, our results demonstrate, for the first time, that adult patients with mastocytosis show increased serum levels of PD-L1. Levels of PD-L1 correlate with severity of disease categories and serum tryptase levels. Furthermore, PD-L1 levels are enhanced in supernatants of cell lines that carry the *KIT* mutation D816V. These findings suggest investigating PD-L1 levels as a diagnostic marker indicating disease progression in adult patients with mastocytosis. Moreover, our data provide a rationale for exploring the efficacy of PD-1 and PD-L1 antibodies in the treatment of advanced mastocytosis.

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Anja Rabenhorst, PhD^a

Silke Leja, MTA^a

Juliana Schwaab, MD^b

Manuela Gehring, MTA^c

Anja Förster, PhD^a

Michel Arock, PharmD, PhD^d

Andreas Reiter, MD^b

Ulrike Raap, MD^c

Karin Hartmann, MD^{a,e}

From ^athe Department of Dermatology, University of Cologne, Cologne, Germany; ^bIII. Medical Clinic, Hematology and Oncology, Medical University of Mannheim, Mannheim, Germany; ^cthe Department of Dermatology and Allergy, Hannover Medical School, Hannover, Germany; ^dMolecular Oncology and Pharmacology, LBPA CNRS UMR8113, Ecole Normale Supérieure de Cachan, Cachan, France; and ^ethe Department of Dermatology, University of Luebeck, Luebeck, Germany. E-mail: karin.hartmann@uksh.de.

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2013064639 A1). U. Raap has received research support and payment for lectures from Novartis Pharma. K. Hartmann has consultant arrangements with Novartis; has received payment for lectures from Abbvie, Biogen, and Novartis; and has received a research grant from Novartis. The rest of the authors declare that they have no relevant conflicts of interest.

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METHODS

Clinical characteristics of patients with mastocytosis and control subjects participating in the study

Levels of PD-L1, PD-L2, and PD-1 were analyzed in the serum of 43 patients with different categories of mastocytosis, classified according to established World Health Organization criteria, and 22 healthy controls. Detailed clinical characteristics of all patients and controls are listed in Table E1. Patients and controls were grouped into adults (10 controls, 31 patients) and children (12 controls, 12 patients). Furthermore, adult patients were grouped on the basis of the disease severity into patients with non-advanced and advanced mastocytosis. Twenty-one patients exhibited non-advanced mastocytosis, and 10 patients belonged to the group of advanced mastocytosis. Within the non-advanced group, 5 patients had mastocytosis in the skin (23.8%) and 16 patients had indolent systemic mastocytosis (76.2%). Within the advanced group, 3 patients were diagnosed with systemic mastocytosis with associated hematological non-MC lineage disease (30.0%), 5 patients with ASM (50.0%), and 2 patients with MCL (20.0%).

Serum measurements in patients with different categories of mastocytosis

The following measurements were performed with patient's serum collected during the last years at the Department of Dermatology, University of Cologne, Cologne, Germany, and at the III. Medical Clinic, Hematology and Oncology, Medical University of Mannheim, Mannheim, Germany. The sera were stored in aliquots at -80°C . Tryptase level was determined by using fluoroimmunoassay (ImmunoCAP Tryptase; Thermo Fisher Scientific, Uppsala, Sweden), and levels of PD-L1, PD-L2, and PD-1 were measured by using ELISA (PD-L1, Cusabio Biotech Co, Wuhan, China; PD-L2, Wuxi Donglin Sci-Tech Development Co, Wuxi/Jiangsu, China; PD-1, Cusabio Biotech Co) according to the manufacturer's guidelines.

Cell lines and measurement of PD-L1, PD-L2, and PD-1 in cell culture supernatants

For *in vitro* experiments, the 2 subclones of the human MC line HMC1 (HMC1.1 and HMC1.2), kindly provided by Dr Joseph H. Butterfield (Mayo Clinic, Rochester, Minn), were cultured in Isocove's modified Dulbecco's medium ($1\times$; Gibco, Invitrogen, Karlsruhe, Germany) containing 10% FBS (Biochrom AG, Berlin, Germany), 2 mM L-glutamine (Biochrom AG), 50 μM 2-mercaptoethanol (Gibco; Invitrogen), 100 U/mL penicillin (Biochrom AG), and 100 $\mu\text{g/mL}$ streptomycin (Biochrom AG). The subclone

HMC1.1 carries 1 *KIT* mutation (V560G), whereas the subclone HMC1.2 carries 2 *KIT* mutations (V560G and D816V).

In addition, ROSA KIT WT and ROSA KIT D816V cells, kindly provided by Dr Michel Arock (Molecular Oncology and Pharmacology, LBPA CNRS UMR8113, Ecole Normale Supérieure de Cachan, Cachan, France), were cultured in Isocove's modified Dulbecco's medium ($1\times$; Gibco; Invitrogen) containing 10% FBS (Biochrom AG), 2 mM L-glutamine (Biochrom AG), 100 U/mL penicillin (Biochrom AG), and 100 $\mu\text{g/mL}$ streptomycin (Biochrom AG). ROSA KIT WT cells are strictly dependent on the continuous presence of 100 ng/mL of recombinant human SCF (PeproTech GmbH, Hamburg, Germany), whereas ROSA KIT D816V cells, carrying the D816V mutation, are independent of growth factors. All cell cultures were maintained at 37°C in 5% CO_2 in a humidified atmosphere.

For measurement of PD-L1, PD-L2, and PD-1 in cell culture supernatants of HMC1.1, HMC1.2, ROSA KIT WT, and ROSA KIT D816V, 1×10^7 cells were incubated in 10 mL of the appropriate medium for 72 hours. Thereafter, supernatants were collected and frozen at -20°C for ELISA measurements according to the manufacturer's guidelines (PD-L1, PD-L2, and PD-1; Wuxi Donglin Sci-Tech Development Co). In 3 separate experiments, each sample was measured twice.

Double immunofluorescence of PD-L1, PD-L2, PD-1, and tryptase

For analysis of PD-L1, PD-L2, PD-1, and tryptase expression in BM and cutaneous lesions of patients with systemic mastocytosis, sections of paraffin-embedded BM and skin biopsies were analyzed by double immunofluorescence staining using the Vectastain ABC-kit (Vector Laboratories, Inc, Burlingame, Calif). Sections were pretreated for antigen retrieval and permeabilization (Vector Laboratories, Inc) before incubation with rabbit anti-PD-L1, anti-PD-L2, or anti-PD-1 antibodies (antibodies-online GmbH, Aachen, Germany) overnight at 4°C . Fluorescein isothiocyanate-coupled goat anti-rabbit antibodies were added as secondary antibodies, and rabbit IgG antibody served as isotype control. Mouse anti-tryptase antibody (abcam, Cambridge, UK), horse anti-mouse biotinylated antibody, and avidin-linked Texas Red dye were applied subsequently. Mouse IgG₁ (R&D Systems GmbH, Wiesbaden, Germany) was used for isotype controls. Analysis was carried out with Zeiss Axiocam and documented with Axiovision software (Carl Zeiss, Microimaging GmbH, Göttingen, Germany).

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 5.01) software using the nonparametric Mann-Whitney *U* test for analysis of serum measurements and the Student *t* test for analysis of cell culture supernatants. Correlation analysis was performed using Spearman rank order correlation. A *P* value of less than .05 was considered statistically significant.

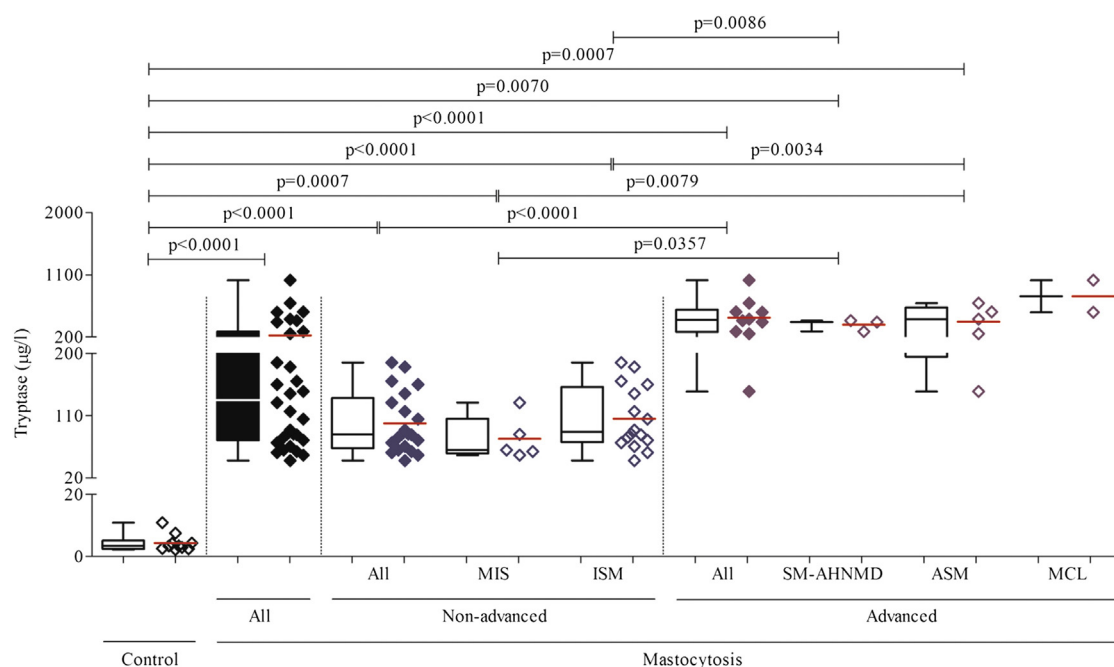


FIG E1. As expected, tryptase serum levels are elevated in adult patients with mastocytosis (All) compared with adult healthy controls (Control). Patients with advanced mastocytosis categories show increased tryptase levels compared with non-advanced categories. Data are presented as *box plots* (min to max) with medians (*left*) and scatter dot plots with means (*right*).

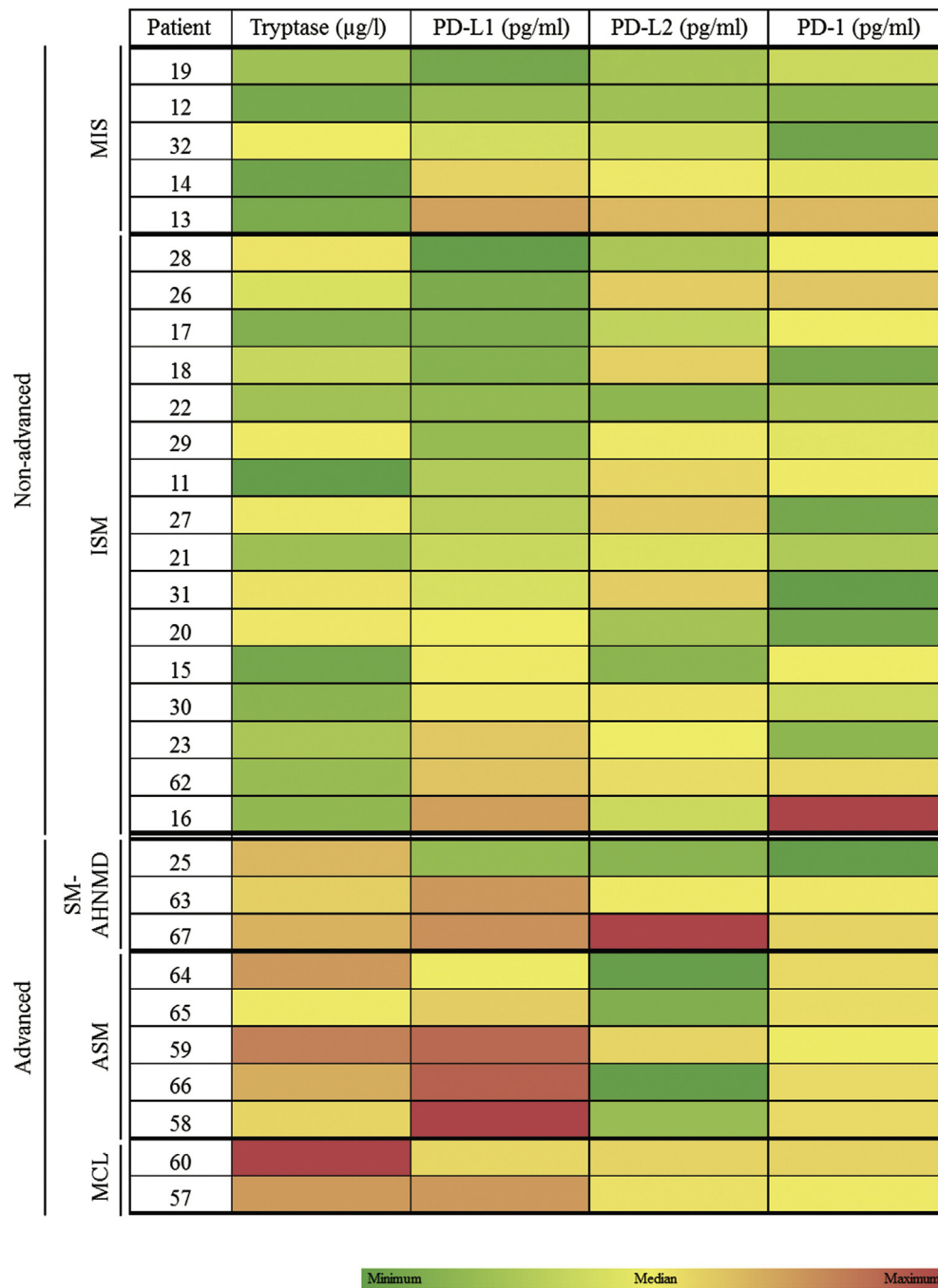


FIG E2. Heat map showing a hierarchical comparison between PD-L1 serum levels and tryptase, PD-L2, and PD-1 serum levels for individual patients. A green-red color scale depicts the amount of measured serum levels (*green*, minimum; *red*, maximum).

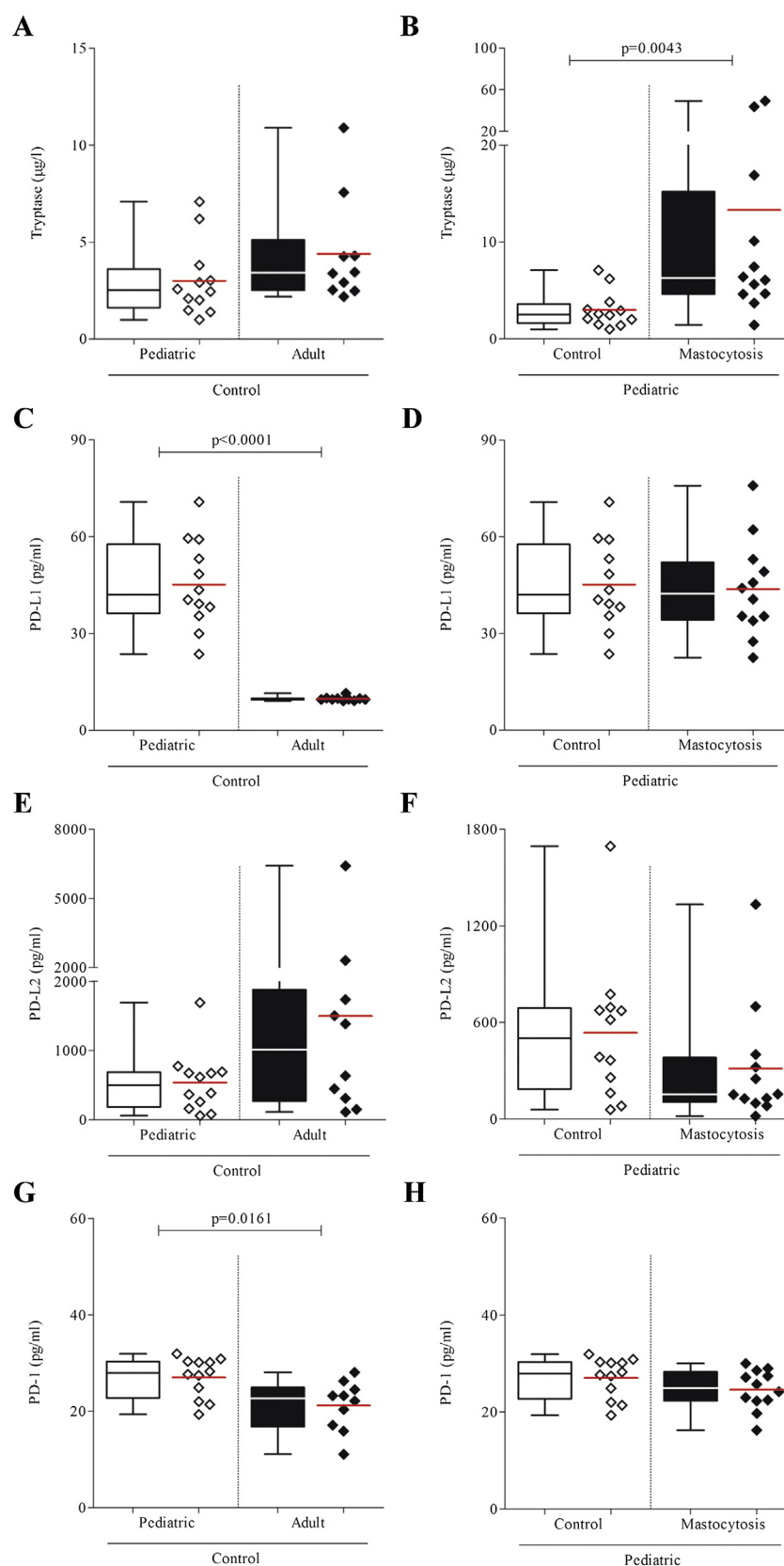


FIG E3. Serum levels of tryptase (**A** and **B**), PD-L1 (**C** and **D**), PD-L2 (**E** and **F**), and PD-1 (**G** and **H**) measured in pediatric patients with mastocytosis (Fig E3, **B**, **D**, **F**, and **H**) and pediatric and adult controls (Fig E3, **A**, **C**, **E**, and **G**). Data are presented as *box plots* (min to max) with medians (*left*) and scatter dot plots with means (*right*).

TABLE E1. Clinical characteristics of patients with mastocytosis and control subjects participating in the study

Parameter	Adult, n (%)			Pediatric, n (%)	
	Control	Mastocytosis		Control	Mastocytosis
		Non-advanced	Advanced		
All	10 (100)	21 (100)	10 (100)	12 (100)	12 (100)
Sex					
Male	3 (30.0)	7 (33.3)	6 (60.0)	6 (50.0)	4 (33.3)
Female	7 (70.0)	14 (66.7)	4 (40.0)	6 (50.0)	8 (66.7)
Age at collection of blood sample (y)					
Mean $\pm$ SD	39.5 $\pm$ 11.8	53.5 $\pm$ 14.8	68.5 $\pm$ 5.2	4.5 $\pm$ 4.0	6.6 $\pm$ 3.5
Range	28-57	29-84	59-76	0-12	2-12
Disease duration (y)					
Mean $\pm$ SD	NA	17.3 $\pm$ 18.1	2.9 $\pm$ 2.8	NA	5.8 $\pm$ 3.1
Range	NA	2-73	0-8	NA	1-12
Tryptase (μ g/L)					
Mean $\pm$ SD	4.4 $\pm$ 2.8	98.8 $\pm$ 43.6	481.4 $\pm$ 250.2	3.0 $\pm$ 1.9	13.3 $\pm$ 16.0
Range	2.92-10.90	45.30-187.00	145.00-1021.00	1.00-7.09	1.44-49.10
Category of mastocytosis					
CM	NA	0 (0)	NA	NA	12 (100)
MIS	NA	5 (23.8)	NA	NA	NA
ISM	NA	16 (76.2)	NA	NA	NA
SM-AHNMD	NA	NA	3 (30.0)	NA	NA
ASM	NA	NA	5 (50.0)	NA	NA
MCL	NA	NA	2 (20.0)	NA	NA

NA, Not applicable.