Increased circulating levels of neurotrophins and elevated expression of their high-affinity receptors on skin and gut mast cells in mastocytosis

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KEY POINTS Patients with mastocytosis feature increased neurotrophin serum levels and elevated expression of modified neurotrophin receptors on skin and gut mast cells. Neurotrophins might contribute to mastocytosis via increased migration of mast cell progenitors, mast cell differentiation, proliferation and/or survival.
ABSTRACT

Mastocytosis is a rare heterogeneous disease characterized by increase of mast cells (MCs) in different organs. Neurotrophins have been shown to promote differentiation and survival of MCs, which in turn represent a major source of neurotrophins. Thus, a contribution of neurotrophins to mastocytosis seems highly conceivable, but has not yet been investigated. We could demonstrate expression of high-affinity neurotrophin receptors TrkA for nerve growth factor (NGF)-β, TrkB for brain-derived neurotrophic factor (BDNF) and NT-4 and TrkC for NT-3 on skin MCs and of TrkA and TrkC on intestinal MCs of patients with mastocytosis. Moreover increased expression of NGF-β, NT-3, TrkA, B, C and isoforms truncated TrkB-T1 and truncated TrkC were observed on skin mast cells. Patients with mastocytosis featured elevated serum levels of NGF, NT-3 and NT-4. Levels of NGF-β and NT-4 correlated with tryptase levels, suggesting a link between mast cell load and blood levels of NGF and NT-4. Migration of CD117+ progenitor cells from the blood was enhanced towards NGF-β gradient in both mastocytosis and controls. Together with enhanced neurotrophin levels, the elevated expression of modified Trk receptors on skin and gut mast cells might contribute to the pathophysiology of mastocytosis in autocrine and paracrine loops.
KEY WORDS

mast cells; mastocytosis; migration; neurotrophins; tropomyosin-related tyrosine kinase receptor
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<td>ASM</td>
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<td>SMC</td>
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INTRODUCTION

Mastocytosis is a rare, heterogeneous disease characterized by an abnormal increase of clonal mast cells (MCs) in one or more organs.\textsuperscript{1} Clinically, cutaneous, gastrointestinal, cardiovascular, neuropsychiatric symptoms and an increased risk of anaphylaxis result from the infiltration of clonal MCs, increased body burden of these MCs, and the release of MC mediators.

Cutaneous mastocytosis (CM) is limited to the skin. Systemic mastocytosis (SM) affects also other organs, mostly the bone marrow (BM) or gastrointestinal tract. Most of the adult patients suffer from indolent SM (ISM) without extracutaneous organ dysfunction. ISM is accompanied by cutaneous involvement, but also manifests as isolated bone marrow mastocytosis (IBMM) without skin involvement, with a favourable prognosis. About 40\% of the patients with SM featured an associated clonal hematological non-MC lineage disease (SM-AHNMD), 12\% an aggressive SM (ASM) and 1\% MC leukemia (MCL) in a recent study.\textsuperscript{2,3} In SM-AHNMD, the prognosis is determined by the associated hematological disease. ASM and MCL are characterized by pronounced organ infiltration with organ dysfunction and cytopenia (C-findings) with a poor prognosis. Smoldering SM (SSM) is a variant of ISM characterized by the presence of \(\geq 2\) B-findings (organomegaly without functional impairment, dysmyelopoiesis, serum tryptase >200 ng/ml and/or pronounced BM >30\%) without C-findings.\textsuperscript{1,3}

Human MCs are derived from CD34\(^+\) bone marrow progenitor cells under the influence of growth factors, particularly stem cell factor (SCF), the ligand for KIT (CD117), which regulate the development of MCs.\textsuperscript{4} Progenitor cells enter the circulation from bone marrow, appearing as mononuclear cells and migrate into peripheral tissues where they differentiate into their final phenotype in response to SCF and, most likely, additional local factors. Mechanisms directing these
progenitors to migrate to and develop in the skin of healthy as well as CM patients, or once mature, to release their mediators, are only partly understood. Gain-of-function mutations in c-kit, the gene for KIT, play a major role in the pathophysiology of mastocytosis, probably leading to the development of greater numbers of MCs and increasing their survival and susceptibility to become activated. However, KIT mutations are not detected in all patients with mastocytosis and have also been found in other neoplastic diseases. Moreover, a clear phenotype-genotype correlation could not be shown yet. Furthermore, mutations in tumor suppressor gene TET2 as well as IL13- and IL4R- polymorphisms and an overexpression of anti-apoptotic proteins Bcl-2/xL have been observed in some patients with mastocytosis.

Therapy of mastocytosis is mostly symptomatic and includes avoidance of trigger factors, therapy of MC mediator release, phototherapy and therapy of osteoporosis, if present. Cytoreductive therapy is applied in advanced forms of SM, especially ASM or MCL. SM-AHNMD, the AHNMD-component of the disease is treated as in patients without SM. To date, no curative treatment for advanced SM exists.

Neurotrophins (NTs) are growth factors, which were initially discovered in the nervous system and play a pivotal role in the development, maintenance and regeneration of nerve fibres. The NTs include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4/-5. NT precursors, pro-NGF/BDNF are high-affinity ligands for the pan-neurotrophin receptor p75 (p75NTR) which together with sortilin promotes apoptosis through the regulation of the rate of p75 cleavage by sortilin, a prerequisite for pro-neurotrophin initiated cell death. Conversely, all mature NTs bind with low affinity to the p75NTR and with high affinity and specificity to the Trk family receptors, TrkA for NGF, TrkB for BDNF and NT-4, and TrkC for NT-3. Each full-length (FL) Trk receptor contains an extracellular,
transmembrane and intracellular domain. Binding of NTs to the extracellular domains of Trk receptors leads to dimerization of the receptors, autophosphorylation of tyrosines in the intracellular domain with activation of downstream signalling pathways including MAPK, PI3K/Akt, PLC-γ and Protein kinase C that favour cellular differentiation, proliferation and survival. Alternative splicing during gene transcription generates receptor isoforms with different functions. Beside the FL receptor, TrkA has two variants, TrkA I and II, with insertions in the extracellular domain, while truncated receptors lacking the intracellular kinase domain have been only discovered for TrkB and TrkC. Although functions of these receptor isoforms are still not clearly understood, accumulating evidence suggests a contribution to cell growth and migration. NTs have also been demonstrated to act as signal transduction molecules between immune cells, structural cells and neuronal cells in allergic diseases.

Neurotrophin-expressing nerves are in direct anatomic contact to MCs and MC-driven skin inflammation has been shown to be impaired in the absence of sensory nerves. NTs have been demonstrated to promote chemotaxis, maturation and survival of MCs which on the other hand produce several NTs. The bidirectional interaction of NTs and MCs suggest a contribution of NTs to a disease such as mastocytosis. However, the role of NTs in mastocytosis has not been investigated sufficiently so far. Therefore, this study aimed to further investigate the role of neurotrophins and their receptors in mastocytosis.
**MATERIAL AND METHODS**

**Study population**

Patients with mastocytosis (n=74; 22 male, 52 female, mean age 50.0±14.4, age range 12-77) and healthy controls (n=50, 19 male, 31 female, mean age 48.8±21.6, age range 20-91) from the Department of Dermatology and Allergy and the Department of Plastic Surgery, University of Bonn, Germany were included in the study after giving their informed consent. Recruitment criteria were a clinically and histologically confirmed mastocytosis in the skin (MIS), CM and/or SM. The group of mastocytosis patients consisted of 14 patients with CM, 2 patients with ASM, 3 patients with SM-AHNMD and 40 patients with ISM, whereof 33 had skin involvement, 4 IBMM and 3 SSM according to the World Health Organizations’s (WHO) criteria.27 WHO criteria for systemic mastocytosis were not assessed in 15 additional patients with MIS because of low serum tryptase, absence of systemic symptoms and/or refusal of bone marrow biopsy.1 Clinical characteristics of the study population are outlined in e-Table 1. A part of the study population has already been described previously.28 The protocol was approved by the local ethics committee and this study was conducted in accordance with the Declaration of Helsinki.

**Tissue processing and identification of KIT mutations** (e-methods 1)

**Reagents and analysis of serum parameters** (e-methods 2)

**Isolation and culture of human SMCs from skin biopsies**

Human SMCs were purified from fresh skin samples and cultured as described previously29,30 (e-methods 3).
Isolation of CD117+ cells from the peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from patients with mastocytosis and from buffy coats of healthy blood donors from the blood bank of the University of Bonn by Lymphoprep gradient technique as described in the manufacturer’s protocol (Axis Shield, Oslo, Norway). CD117+ cells were isolated from PBMCs by immunomagnetic selection with mAb against human CD117 (c-kit) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer’s instructions with some minor variations. The purity of CD117+ cells was >90% as measured by flow cytometric staining.

Flow cytometry

Flow cytometry was performed as described previously. Finally, the cells were measured with FACS-Canto (BD Biosciences, Heidelberg, Germany) and analysed by FACSDiva (BD Biosciences, Heidelberg, Germany) and FlowJo (TreeStar Inc., Ashland, OR, U.S.A) software.

RNA isolation and real time PCR

mRNA from cultured SMCs was isolated with the NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany) including digestion of genomic DNA and was subjected to cDNA synthesis with TaqMan reverse transcription reagents with random hexamers according to the manufacturer’s instructions (Applied Biosystems, Darmstadt, Germany). The prepared cDNA was amplified using TaqMan assay Master Mix (Applied Biosystems) according to the recommendations of the manufacturer in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Expression of TrkA (Hs01021018_m1), TrkB (Hs01093103_m1), TrkC (Hs00983870_m1), NGF-β (Hs00171458_m1), NT-3 (Hs00267375_s1) and NT-4/5 (Hs01596132_m1) in relation
to 18s rRNA endogenous control (4310893E) was evaluated using TaqMan Assays (Applied Biosystems). Relative quantification and calculation of the range of confidence was performed using the comparative \( \Delta \Delta CT \) methods.\(^{32}\) All analyses were conducted in duplicates.

Detection of transcript variants of Trk receptors with PCR

To analyze the expression of transcript variants of TrkA, B and C on cultured SMCs from patients with mastocytosis and controls, primers were synthesized (Microsynth, Balgach, Switzerland) (Supplementary Table 1) and PCRs performed as described previously.\(^{9,12,33}\) The amount of human template cDNA in different PCRs was determined with a parallel PCR for a 114bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene with following primers, GAPDH\_forward: CCA CAT CGC TCA GAC ACC AT and GAPDH\_reverse: GGC AAC AAT ATC CAC TTT ACC AGA GT.\(^{11}\) In each PCR, human brain cDNA and distilled water were used as positive and negative control, respectively.

Immunohistochemistry staining

Immunohistochemistry was performed as described previously\(^{34}\) using serial paraffin embedded sections (4\( \mu \)m) with the help of mAbs to TrkA, B and C from R&D Systems and toluidine blue. Appropriate isotype-matched controls were included. TrkA, B, C and toluidine blue positive cells were counted in 600 consecutive fields per section (300 upper dermis, 300 lower dermis; magnification x200, field diameter 0.05 mm\(^2\), total area 30 mm\(^2\)) under microscope (BH-2 microscope and DF70 camera, Olympus Europe GmbH, Hamburg Germany) and the mean of Trk\(^+\) or toluidine blue\(^+\) cells/mm\(^2\) was calculated.
**Immunofluorescence staining**

Immunofluorescence staining was performed on 4-µm skin and gut sections of paraformaldehyde-fixed and paraffin-embedded tissue as described elsewhere.\textsuperscript{35} Sections were treated with mAb against tryptase (Dako, Hamburg, Germany) at 4°C overnight. TrkA, B, C was analyzed with polyclonal rabbit antibodies or appropriate isotype-matched control. Antibody binding was detected with appropriate anti-mouse and anti-rabbit Cy2 and Cy3 labelled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were analyzed with a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using Diskus and ImageJ software (National Institute of Health Web site) for documentation and analysis.

**ELISAs** (e-methods 4)

**Migration assays**

Cell migration was performed in 24-well Transwell cell culture chambers (Costar, Corning, NY) in accordance with published protocols\textsuperscript{36} with some minor variations (e-methods 5).

**Statistical analysis**

Differences between the groups were assessed using the Chi\textsuperscript{2} test for qualitative parameters, Mann-Whitney-U test for neurotrophins, tryptase and IgE ELISA data, and the Spearman-Rho test for correlations. Real-time PCR data were analyzed with the t-test. Log-transformed migration data were analyzed using repeated measurement ANOVA with Bonferroni-corrected pairwise comparison of conditions.
for both groups and paired-sampled t-test, when looking at separate groups (SPSS 21.0, SPSS Inc., Chicago, Illinois). Results are given as mean ± standard deviation (SD) or standard error of the mean (SEM), respectively. Any $P$-values are two-sided and <0.05 considered statistically significant. Except for the repeated measurement ANOVA, no correction for multiple testing was made for other descriptive tests and $P$-values.
RESULTS

Increased serum levels of NGF-β, NT-3 and -4 in patients with mastocytosis

We observed significantly increased serum levels of NGF-β (P=.04), NT-3 (P=.04), NT-4 (P=.002) in patients with mastocytosis compared to controls (Figure 1). NGF-β levels were highest in patients with IBMM, but did only reach statistical significance in patients with ISM with skin involvement (P=.004) and all patients with ISM (P=.001). Conversely, patients with a pure CM had the highest levels of BDNF, NT-3 (P=.027) and similar to MIS of NT-4 (P=.045), but also patients with ISM showed significantly higher levels of NT-4 (P=.02) compared to controls. In the analysis of the separate subgroups of ISM, only in patients with ISM and skin involvement, a narrow significance was reached (P=.048), but not in patients with SSM or IBMM. There were no significant differences between BDNF levels of patients with mastocytosis compared to controls or between NT levels of the different mastocytosis variants.

NT levels correlated among each other. NGF-β showed a positive correlation to NT-3 ($r_s=0.48$, $P=7.8\times10^{-6}$) and BDNF ($r_s=0.35$, $P=.004$). Conversely, NT-4 correlated negatively with NGF-β ($r_s=-0.21$, $P=.004$) and NT-3 ($r_s=-0.25$, $P=.02$). Interestingly, NGF-β ($r_s=0.35$, $P=4.5\times10^{-4}$) and NT-4 ($r_s=0.31$, $P=.002$) serum levels correlated with serum tryptase levels, implying a direct link between MC load and NGF-β and NT-4 blood levels in patients with mastocytosis. We did not observe significant differences in NT levels between mastocytosis patients carrying an activating KIT mutation compared to those without, analyzed in a subgroup of n=24 patients (data not shown).
mRNA expression of NGF-β, NT-3, TrkB and TrkC is increased in SMCs of patients with mastocytosis

Patients with mastocytosis exhibited a higher mRNA expression of NGF-β ($P=.05$), NT-3 ($P=.02$), NT-4/5 (n.s.), TrkB ($P=.04$), and TrkC ($P=.03$), but only slightly increased TrkA (n.s.) mRNA expression in cultured SMCs (Figure 2) compared to controls.

Increased infiltration of MCs expressing TrkA, TrkB and TrkC in the dermis and of mast cells expressing TrkA and TrkC in the gut of patients with mastocytosis

Increased infiltration of MCs in the dermis of mastocytosis patients was demonstrated by toluidine blue staining of lesional skin (Figure 3A-C). Immunohistochemical staining of those skin sections revealed significantly higher numbers of dermal cells expressing TrkA, TrkB and TrkC in mastocytosis patients compared to controls (Figure 3D-L).

To determine, whether the TrkA$^+$, TrkB$^+$ and TrkC$^+$ cells are MCs, immunofluorescence double staining was performed. TrkA$^+$, TrkB$^+$ and TrkC$^+$ cells in patients with mastocytosis spatially overlaid with cells expressing tryptase, demonstrating that most of the cells expressing Trk receptors in the dermis were tryptase$^+$ SMCs (Figure 4A-C). Furthermore, TrkA and C positive but TrkB negative tryptase$^+$ MCs were detectable in the gut (Figure 5) of patients with systemic mastocytosis. All together, immunofluorescence double staining confirmed infiltration of skin with TrkA$^+$, TrkB$^+$ and TrkC$^+$, tryptase$^+$ MCs and gut with TrkA$^+$, TrkB$^-$ and TrkC$^+$, tryptase$^+$ MCs.
Enhanced expression of receptor isoforms of TrkB and TrkC on human SMCs from patients with mastocytosis

Human SMCs from both patients with mastocytosis and controls express TrkA mRNA, which contains highly conserved regions of the extracellular domain as well as the tyrosine kinase domain (Figure 6A) suggesting functional TrkA receptors. Human SMCs expressed exclusively the exon lacking TrkAI splice variant (Figure 6A, 102bp)-supposed to be less responsive to NT-3 than TrkAII. Both FL and truncated TrkB mRNA were expressed in SMCs from patients with mastocytosis. Sequence determination confirmed expression of the TrkB isoforms (data not shown). Mastocytosis patients had a different gene expression profile of TrkB on human SMCs compared to controls. Exons 1-4 and major part of exon 5 encode the conventional 5’ untranslated region (UTR) of the human TrkB gene and serve as transcription start sites. We detected higher expression of 5’- UTR gene in SMCs of mastocytosis patients (e-Figure 1). SMCs from mastocytosis patients and controls had same mRNA expression level of exon 24, which encodes FL-TrkB receptor containing the tyrosine kinase domain (Figure 6B, exon 21-24). PCR results indicated that both FL-TrkB and a receptor variant without exon 22 are expressed by human SMCs.

TrkB-T1-mRNA expression on SMCs from mastocytosis patients was increased compared to controls (Figure 6B, exon 15-16). Other truncated TrkB isoforms, such as TrkB-TK and TrkB-T-Shc employing exon 22b and exon 19 respectively, were not detected. Moreover, recently identified TrkB receptor utilizing exon 5c was not expressed on human SMCs. Notably, SMCs from mastocytosis patients had higher mRNA expression of extracellular domain of TrkB than controls (Figure 6B, exon 5-11). A schematic picture of TrkB-FL and TrkB-T1 isoforms identified on human SMCs according to TrkB PCR data illustrates expression of TrkB on human SMCs (Figure...
6D). Finally, SMCs of both mastocytosis patients and controls expressed the truncated TrkC isoforms lacking the tyrosine kinase domain (Figure 6C).

**NGF-β increases migratory properties of peripheral CD117+ cells via TrkA**

Migration of CD117+ progenitor cells from blood of both patients with mastocytosis ($P=.003$) and controls ($P=2.3\times10^{-6}$) was significantly increased towards NGF-β ($P=1.7\times10^{-5}$ for all subjects), but not towards a BDNF, NT-3 and NT-4 gradient (Figure 7A) and did not differ significantly between mastocytosis patients and controls. Migration towards NGF-β was significant higher compared to migration to BDNF, NT-3 and NT-4 in both mastocytosis patients ($P=.004; .02; .03$, respectively), and controls ($P=.001; 7.8\times10^{-5}; 3.8\times10^{-4}$, respectively). The number of migrated cells increased dose-dependently (Figure 7B). Addition of anti-human TrkA antibody specifically blocking cell-surface TrkA mediated activity significantly prevented migration of CD117+ cells towards NGF-β in mastocytosis patients ($P=.03$) and controls ($P=.009$) ($P=.002$ for all subjects), indicating that NGF-β driven migration of CD117+ cells was mainly mediated via TrkA (Figure 7C).
DISCUSSION

Here we report for the first time the expression of TrkA, B and C on human SMCs and an increased expression on SMCs of mastocytosis patients. Intestinal MCs from patients with mastocytosis expressed TrkA and C, but not TrkB. We observed a significant increase of cells expressing NGF high-affinity receptor TrkA in affected skin lesions of mastocytosis patients, which could be identified as MCs by immunofluorescence staining and flow cytometry. Human SMCs from both mastocytosis patients and controls expressed TrkA mRNA, which contains highly conserved regions of the extracellular domain as well as the tyrosine kinase domain suggesting functional TrkA receptors.

Our results indicate that human SMCs express exclusively the TrkAI splice variant, but not TrkAll. These results, together with (i) increased NGF and NT-3 mRNA expression in SMCs and (ii) the correlation of NGF-β and NT-4 serum levels with serum tryptase, strongly suggest MCs themselves might serve as source for the elevated circulating NT serum levels observable in patients with mastocytosis.

Previously, NTs have also been shown to exert chemotactic effects on fibroblasts, NGF on RPMCs and melanocytes. In line with those findings, we demonstrated a significantly increased migration of peripheral CD117+ cells towards a NGF-β gradient via TrkA, which was expressed on MCs of skin and gut, organs frequently infiltrated in mastocytosis. The expression of TrkA and TrkC on human intestinal MCs is consistent with previous reports, which observed TrkA and TrkC expression on the mRNA and protein level, while demonstration of TrkA, TrkB and TrkC on skin MCs in general and of NT receptors in mastocytosis is novel.

Most interestingly, the human leukaemia mast cell line-1 (HMC-1) has been shown to express TrkA, TrkB, and TrkC receptor proteins containing full-length tyrosine kinase domains as well as TrkAI and truncated TrkB and TrKC. NGF has been shown to
induce upregulation of early growth response genes and other proliferation and survival signals in HMC-1 via TrkA \(^9,18-20\) and to rescue HMC-1 (V560G c-kit) cells from cell death mediated by IM, NGF together with SCF\(^23\) could also promote growth, differentiation and survival of cord-blood-derived MCs (CBMCs)\(^9,19,20,23\) and murine MCs.\(^25,41\) Thus, increased NGF serum levels together with elevated expression of NGF and TrkA on SMCs of mastocytosis patients and an increased migration of CD117\(^+\) cells from the blood toward a NGF gradient via TrkA might contribute to the increased numbers of MCs via increased migration and proliferation of mast cell progenitors.

As also several other skin cells such as fibroblasts, keratinocytes, melanocytes, lymphocytes, macrophages, endothelial cells, eosinophils and cutaneous nerve fibres have been shown to produce NTs,\(^16,38,39,41\) the skin represents an environment rich in NTs which might attract the migration of MC progenitor cells. A higher level of NGF-β in sera of patients with IBMM without skin lesions might be of pathogenic significance as it would create less of a gradient between skin and intravascular compartment and discourage mast cell progenitors to migrate to the skin. However, the number of patients with IBMM was too small to allow further conclusions and there was no significant difference in NGF serum levels between patients with CM or MIS compared to those with IBMM.

Furthermore, we detected a significantly higher expression of TrkB and TrkC on SMCs of mastocytosis patients both on the mRNA and protein level as well as increased levels of their ligands NT-3 and NT-4. TrkB receptors exist as a full length isoform (FL-TrkB) and three different truncated isoforms (TrkB-T1, TrkB-T-TK and TrkB-Shc).\(^9-12\) Previously, the expression of TrkAI, truncated TrkB and truncated TrkC could be shown on HMC-1 cells.\(^9\) Our PCR results demonstrated that human SMCs express both FL-TrkB and truncated TrkB-T1, but not TrkB-T-TK and TrkB-T-
Shc. Higher expression of 5'- UTR gene in SMCs of patients with mastocytosis suggests higher activity of gene transcription. Interestingly, compared to controls, SMCs from mastocytosis patients had much higher expression levels of truncated TrkB-T1 and genes encoding the extracellular domain of TrkB, while the transcription of the intracellular domain of the FL-TrkB was not impacted. Because both FL- and truncated receptors need the extracellular domain to form the complete receptor, less expression of the extracellular domain in human SMCs could be the limiting step for transcription of both FL-TrkB and truncated TrkB-T1. The biological functions of FL-TrkB and truncated TrkB and their cooperation on human MCs are still largely unknown. Studies investigating the development of the nervous system and tumorigenesis indicate an essential role of Trk receptors for cell proliferation and tissue development. Tumor patients with high expression of TrkB are regarded to have a poor prognosis.13,42 FL-TrkB is preferentially expressed during early stages of embryogenesis, and replaced by truncated TrkB-T1 during postnatal development.43,44 The truncated TrkB-T1 consists of a transmembrane and extracellular domain, which binds its natural ligands, while its short intracellular tail does not have any tyrosine kinase activity. TrkB-T1 has long been regarded as a negative regulator of BDNF signaling via forming heterodimers with FL-TrkB.45 However, recent studies indicate that TrkB-T1 is a functional receptor which is capable of mediating intracellular signaling cascades.46-47 Here, the identification of FL-TrkB expression and elevated expression of truncated TrkB-T1 on SMCs from mastocytosis patients indicates an abnormal status of TrkB expression in this disease and the putative pathophysiological role of it has to be elucidated.

TrkA and TrkC have recently been demonstrated to act as dependence receptors, which induce signals for survival, differentiation and migration in the presence of their ligand and apoptosis in the absence of their ligand NGF-β and NT-3.42 For both
ligands, we could show increased circulating levels as well as increased expression on SMCs in patients with mastocytosis. In human intestinal MCs, NT-3, but not NGF, has been shown to promote proliferation and to reduce apoptosis of human intestinal MCs via TrkC together with SCF. Conversely, increased numbers of MC have been observed in the neonatal skin of NT-3-overexpressing mice, without showing a higher rate of MC proliferation. However, the expression and presumably also functions of NTs and their high affinity receptors for NTs differs between MCs from humans and mice and also between different human tissues and human cell lines.

In this study, we could demonstrate for the first time significantly higher circulating levels of the NTs NGF-β, NT-3 and NT-4 and increased migration of CD117+ cells from the blood towards a NGF gradient via TrkA. NTs might contribute to the augmented MC tissue infiltration in mastocytosis via increased migration of MC progenitor cells towards NGF and/or stimulation of MC differentiation and proliferation by NGF and NT-3. As MC themselves represent a source of neurotrophins with functional receptors, they might augment these effects in an autocrine feedback loop.

In conclusion, our data indicate a pivotal role of neurotrophins in the pathophysiology of mastocytosis. The sophisticated network between MC progenitors in the blood, MCs in the skin and soluble mediators of the nervous system such as NTs might be of major relevance not only for the understanding of mechanism triggering mastocytosis, but also other MC-driven diseases. Further on, better knowledge about factors promoting MC infiltration might help to identify structures, which could be efficiently targeted in the context of therapeutic approaches, aimed at attenuating unwanted over-activation and tissue infiltration of MCs. Several Trk inhibitors are currently being developed with some small molecule inhibitors already being in phase
1 and 2 clinical trials\textsuperscript{13,42}, which might potentially also present a therapeutical option for mastocytosis in the future.
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### AUTHORS CONTRIBUTIONS

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### Conflict of interest:

Authors declare no conflict of interest.


34. Pauls K, Merkelbach-Bruse S, Thal D, Buttner R, Wardelmann E. PDGFRalpha- and c-kit-mutated gastrointestinal stromal tumours (GISTs) are characterized by distinctive histological and immunohistochemical features. *Histopathology* 2005;46(2):166-175.


FIGURE LEGENDS

Figure 1. Increased serum levels of NGF-β, NT-3/4 in patients with mastocytosis

Nerve growth factor (NGF)-β (A), brain-derived neurotrophic factor (BDNF) (B), Neurotrophin (NT)-3 (C) and NT-4 (D) serum levels of healthy controls (CTR) (n=50 for NGF-β, NT-4; n=33 for BDNF; n=40 for NT-3) and patients with mastocytosis (MAST) (n=49 for NGF-β; NT-4; n=33 for BDNF, n=39 for NT-3). ASM=Aggressive systemic mastocytosis (n=2 for NGF-β; NT-3; NT-4, n=1 for BDNF), SM-AHNMD=systemic mastocytosis with an associated clonal hematological non-MC lineage disease (n=1 for all NTs), MIS=mastocytosis in the skin (n=11 for NGF-β, NT-4; n=6 for BDNF; n=5 for NT-3); CM=Cutaneous mastocytosis (n=8 for NGF-β, NT-4; n=6 for BDNF; n=6 for NT-3); ISM=indolent systemic mastocytosis (n=27 for NGF-β; NT-4; n=25 for NT-3, n=19 for BDNF), ISM*=indolent systemic mastocytosis with skin involvement (n=20 for NGF-β; NT-4; n=19 for NT-3, n=13 for BDNF), SSM=smoldering systemic mastocytosis (n=3 for all NT), IBMM=isolated bone marrow mastocytosis (IBMM) (n=4 for NGF-β, NT-4; n=3 for BDNF, NT-3).

Figure 2. Elevated NGF, NT-3, TrkB and TrkC mRNA expression on skin mast cells from patients with mastocytosis

Relative mRNA expression of NGF-β (A), NT-3 (B), NT4/5 (C) and TrkA (D), TrkB (E), TrkC (F) on cultured SMCs isolated from healthy skin (CTR, n=5 for all except of NT-4/5 (n=4)) or patients with mastocytosis (MAST; n=5 including CM (n=1) and ISM (n=4 for each group except NT-4/5 (n=3)) are depicted, mean values ± SEMs are shown.
Figure 3. Increased number of MCs correlates with upregulated number of TrkA, TrkB and TrkC positive cells infiltrating the dermis in cutaneous mastocytosis

Toluidine blue (A-C), Anti-TrkA (D-F), Anti-TrkB (G-I), Anti-TrkC (J-L) staining of skin biopsies of healthy controls and patients with mastocytosis (lesional skin). Immunohistochemistry stainings of a representative paraffin section of healthy skin (A, D, G, J), cutaneous mastocytosis (B, E, H, K) and mean values and SEMs of the number of cells in the upper (left side) and lower (right side) dermis of 10 controls and 10 patients with mastocytosis (C, F, I, L; CM (n=5); ISM (n=4); SM-AHNMD (n=1) are depicted.

Figure 4. Immunofluorescence double staining identifies TrkA⁺, TrkB⁺ and TrkC⁺ cells infiltrating the skin of patients with mastocytosis as MCs

A-C: Representative immunofluorescence double staining of skin biopsy taken from a healthy control (CTR) and a patient with mastocytosis (MAST) with an anti-tryptase antibody labelled with Cy-2 (green) and anti-TrkA, anti-TrkB and anti-TrkC antibodies labelled with Cy-3 (red) and the overlay pictures of both stainings are shown.

Figure 5. TrkA⁺ and TrkC⁺ mast cells infiltrate the gut of patients with systemic mastocytosis.

Representative immunofluorescence double staining of gut sections (magnification x400) of patients with mastocytosis (n=4) with an anti-tryptase antibody labeled with Cy-2 (green) and anti-TrkA, anti-TrkB and anti-TrkC antibodies labeled with Cy-3 (red), overlay pictures of both stainings are shown, white bar=50 μm.
Figure 6. Expression patterns of Trk receptors on human SMCs from patients with mastocytosis and healthy controls

A: Full-length TrkA and alternatively spliced trkA mRNA isoforms in human SMCs. B: Expression of TrkB transcripts in human SMCs. Amplified exons of TrkB receptor are depicted on the right side and names of alternative TrkB isoforms are shown on the left. C: Expression of tyrosine kinase domain (TK1: exons 13-14; TK2: exons 15-17) and truncated isoforms of TrkC in human SMCs. Representative PCR results of cDNAs from SMCs of patients with mastocytosis (MAST, n=13: CM (n=5); ISM (n=7), SM-AHNMD (n=1)) and healthy individuals (CTR, n=14) are shown. D: Schematic drawing of TrkB-FL and TrkB-T1 mRNA and protein isoforms expressed on human SMCs. Exons are shown as boxes and introns are shown as lines. C, cysteine rich region; Leu rich, leucine rich region; IG like, immunoglobulin like-domain; SHC, Shc-binding domain; and PLC-γ, PLC-γ binding domain.

Figure 7. NGF-β-enhanced migratory properties of CD117+ cells from the blood

A: Relative change of the migratory activity of human peripheral CD117+ progenitor cells from healthy controls (n=12 for NGF, n=6 for BDNF, NT-3, NT-4) and patients with mastocytosis (n=6, CM (n=1), ISM (n=4), SSM (n=1)) towards a NGF-β, BDNF, NT-3 and NT-4 gradient in transwell chamber experiments in comparison to unstimulated controls (CTR) is shown as mean values and SEMs. B: Number of human peripheral migrated CD117+ progenitor cells from healthy controls (n=4) towards a NGF-β gradient increases dose-dependently. C: Addition of anti-human TrkA antibody significantly prevented migration of CD117+ cells towards NGF-β in both patients with mastocytosis and healthy controls (n=6 each), indicating that NGF-β driven migration of CD117+ cells was mainly mediated via TrkA.
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7

A

B

C

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Increased circulating levels of neurotrophins and elevated expression of their high-affinity receptors on skin and gut mast cells in mastocytosis

Wenming Peng, Laura Maintz, Jean Pierre Allam, Ulrike Raap, Ines Gütgemann, Jutta Kirfel, Eva Wardelmann, Sven Perner, Wei Zhao, Rolf Fimmers, Klaus Walgenbach, Johannes Oldenburg, Lawrence B. Schwartz and Natalija Novak