DISSERTATION

Characterization of the role and relevance of mas-related G protein-coupled receptor X2 expression in the skin of patients with mycosis fungoides

Charakterisierung der Rolle und Relevanz der Expression von *mas-related G protein-coupled receptor X2* in der Haut von Patienten mit Mycosis fungoides

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List of abbreviations

BMI	Body mass index
BSA	Body surface area
CBCL	Cutaneous B cell lymphoma
CST	Cortistatin
CTCL	Cutaneous T-cell lymphoma
DAPI	4',6-diamidinyl-2-phenylindole
DLQI	Dermatological life quality index
IgE	Immunoglobulin E
IL-31	Interleukin-31
ItchyQol	Itch-specific quality of life questionnaire
MCs	Mast cells
MF	Mycosis fungoides
MRGPRs	Mas-related G protein-coupled receptors
MRGPRX2	Mas-related G protein-coupled receptor X2
QoL	Quality of life
scRNAseq	Single-cell RNA sequencing
SWAT	Severity-weighted assessment tool
VAS	Visual analogue scale

Abstract

Cutaneous T-cell lymphoma (CTCL) encompasses a range of lymphoproliferative disorders characterized by the clonal accumulation of malignant T-lymphocytes in the skin, with Mycosis fungoides (MF) being the most prevalent subtype. Patients with primary CTCL often experience severe pruritus, which significantly diminishes their quality of life (QoL). Despite this, the full impact of CTCL-associated pruritus is not yet fully understood, and, importantly, many patients show resistance to conventional anti-pruritic therapies such as topical steroids or oral antihistamines, highlighting the need for a deeper understanding of the mechanisms of pruritus in CTCL.

Recent research has identified the mas-related G protein-coupled receptor X2 (MRGPRX2) as an important mast cell (MC) receptor implicated in IgE-independent non-histaminergic itch. However, the significance of MRGPRX2 in CTCL has not been thoroughly investigated. The aim of this dissertation is to characterize in detail the pruritus experienced by CTCL patients, including itch severity and effects on patients' QoL, and to explore the underlying mechanisms of pruritus in CTCL with the goal of identifying potential novel therapeutic targets.

To achieve these objectives, I have gathered clinical data, including evaluations of itch intensity, and collected skin biopsies from ten MF patients and eight healthy individuals. Immunohistochemical staining of MRGPRX2 was performed on both affected and unaffected MF skin and healthy skin samples, complemented by immunofluorescence staining to detect the co-localization of MRGPRX2 and the MC marker tryptase. Additionally, publicly available single-cell RNA sequencing data from MF patients and healthy controls was reanalyzed to pinpoint MRGPRX2 expression across various cell populations.

The findings presented in my dissertation indicate that pruritus (itch) is a prevalent symptom affecting 70% of MF patients. A correlation was established between the severity of pruritus and both a reduction in QoL and an escalation in disease severity among MF patients. An elevation in MRGPRX2-positive cells was observed in the affected skin of MF patients compared to unaffected and healthy skin, with MCs being the predominant cell type exhibiting MRGPRX2 expression in MF patients. Additionally, the proportion of

MRGPRX2-positive MCs to the total MRGPRX2-positive cells in both affected and unaffected skin exhibited a positive correlation with the severity of the disease.

In summary, pruritus constitutes a significant clinical concern in MF, a variant of CTCL, necessitating effective management strategies. My dissertation points to MRGPRX2 and MCs as potential key players in MF pathogenesis and advocates for continued research. The insights presented here could potentially lead to the identification of novel therapeutic targets in the management of pruritus in MF patients.

Zusammenfassung

Das kutane T-Zell-Lymphom (CTCL) umfasst eine Reihe von lymphoproliferativen Erkrankungen, die durch Akkumulation klonaler T-Zellen in der Haut gekennzeichnet sind, mit der Mycosis fungoides (MF) als häufigster Subtyp. CTCL patienten erleben oft intensiven Juckreiz, dessen Bedeutung noch immer nicht vollständig geklärt ist, obwohl viele Patienten auf Therapien wie topische Steroide oder orale Antihistaminika nicht ansprechen. Dies betont die Notwendigkeit, die Mechanismen des Juckreizes bei CTCL besser zu verstehen.

Jüngste Forschungen haben den *mas-related G protein-coupled receptor X2* (MRGPRX2) als einen wichtigen Mastzellrezeptor identifiziert, der für IgE-unabhängigen, nichthistaminergen Juckreiz verantwortlich ist. Die Rolle von MRGPRX2 bei CTCL ist bislang nicht bekannt. Ziel dieser Dissertation ist es, den Juckreiz von CTCL-Patienten genauer zu charakterisieren und neue Erkenntnisse über zugrunde liegenden Mechanismen des Pruritus zu gewinnen.

Hierfür wurden zahlreiche klinische Daten gesammelt und Hautbiopsien von zehn MF-Patienten und acht gesunden Personen untersucht. Die Expression von MRGPRX2 wurde mittels Immunhistochemie an läsionaler und gesunder Haut von MF Patienten und gesunden Probanden durchgeführt, ergänzt durch Immunfluoreszenzfärbung zum Nachweis der Ko-Lokalisierung von MRGPRX2 und dem Mastzell-Marker Tryptase. Zusätzlich wurden öffentlich verfügbare Einzelzell-RNA-Sequenzierungsdaten von MF-Patienten und gesunden Kontrollen re-analysiert, um die MRGPRX2 Expression in verschiedenen Zellpopulationen zu bestimmen.

Die Ergebnisse zeigen, dass Pruritus ein weit verbreitetes und relevantes Symptom ist, an dem 70 % der MF-Patienten leiden. Es wurde eine Korrelation zwischen der Schwere des Juckreizes und der Einschränkung der Lebensqualität sowie einer Verschlechterung der Krankheitsschwere bei MF-Patienten festgestellt. In läsionaler Haut von MF-Patienten wurde im Vergleich zu nicht betroffener und gesunder Haut eine erhöhte Zahl an MRGPRX2-positiven Zellen gefunden, wobei Mastzellen der vorherrschende Zelltyp mit MRGPRX2-Expression bei MF-Patienten waren. Darüber hinaus wies der Anteil der MRGPRX2-positiven Mastzellen an den gesamten MRGPRX2-positiven Zellen sowohl in der betroffenen als auch in der nicht betroffenen Haut eine positive Korrelation mit dem Schweregrad der Erkrankung auf.

Zusammenfassend stellt Juckreiz bei MF eine erhebliche klinische Herausforderung dar, welche eine wirksame Behandlungsstrategie erfordert. Meine Dissertation weist auf MRGPRX2 und Mastzellen als potenzielle Schlüsselelemente in der Pathogenese von MF hin und plädiert für weiterführende Forschung in diesem Bereich. Die hier vorgestellten Erkenntnisse könnten möglicherweise zur Identifizierung neuer therapeutischer Ziele für die Behandlung von Pruritus bei MF-Patienten führen.

1 Introduction

1.1 Pruritus in mycosis fungoides is highly prevalent and difficult to treat

1.1.1 Categorization of cutaneous lymphomas

As per its definition, primary cutaneous lymphomas are a subclass of non-Hodgkin lymphomas that initially present in the skin without signs of extracutaneous disease at the moment of diagnosis [1]. They account for 19% of all extranodal non-Hodgkin lymphomas, ranking just behind gastrointestinal lymphomas in frequency [2-4]. These lymphomas can eventually spread to involve lymph nodes, the circulatory system, and other organs [4]. They are broadly divided into cutaneous T cell lymphoma (CTCL) and cutaneous B cell lymphoma (CBCL) subtypes, with CTCL constituting approximately 75-80% of all cutaneous lymphomas on a global scale [1].

Within all of CTCL, mycosis fungoides (MF) stands as the most common and classic subtype, encompassing around 50% of all primary cutaneous lymphomas and about 60% of CTCL patients [1]. MF has several variants, including pagetoid reticulosis, folliculotropic MF, and granulomatous slack skin [2, 5]. In contrast, Sézary syndrome, another classic CTCL subtype, is far less common, accounting for less than 3% of all CTCL cases and predominantly affecting adults [1]. First described by Sézary and Bouvrain in 1938, Sézary syndrome is distinguished by erythroderma, generalized lymphadenopathy, and the presence of malignant T cells (Sézary cells) in the skin, lymph nodes, and blood [6, 7]. Sézary syndrome is a more aggressive leukemic and erythrodermic form of CTCL with a poor prognosis [4, 8] and the median survival period is 2-4 years [4, 6].

On the other hand, CBCL constitutes roughly 20-25% of all primary cutaneous lymphomas and is further classified into three primary types: marginal zone B-cell lymphoma, follicle center lymphoma, and diffuse large B-cell lymphoma [1, 3]. Although approximately 40% of CBCL cases report localized itch [9], the clinical concern of itch appears to be less significant in CBCL compared to CTCL. Considering the rarity of CBCL relative to CTCL, and the specific interest in pruritus within these investigations, I focused in my thesis on CTCL.

1.1.2 Epidemiology of mycosis fungoides

The incidence of MF has steadily risen over recent decades, though the reasons behind this increase remain unclear [10]. In the United States, cases have climbed from 3.0 to 5.9 per million people from the 1970s to the 2010s [11]. This disease predominantly affects older adults, with most patients being around 55 to 60 years old at the time of diagnosis [4]. Furthermore, there is a significant male predominance, with a male-to-female ratio of 1.6-2.0:1 [4].

1.1.3 Clinical features of mycosis fungoides

MF usually progresses through three distinct phases: the patch stage, plaque stage and tumor stage [4, 12]. In the early phase of MF, characteristic lesions manifest as erythematous macules or papules. In the tumor phase of MF, lesions typically exhibit a combination of patches, plaques, and tumors that may ulcerate. Erythrodermic MF can affect nearly the entire skin surface, leaving only small areas unaffected [7]. However, MF often mimics various inflammatory and infectious skin conditions due to its diverse clinical and pathological manifestations. The atypical nature of MF presentations can pose challenges for diagnosis, necessitating a high degree of suspicion and thorough clinicopathologic correlation [7].

Prognosis and staging at diagnosis are closely linked in MF [13]. Early-stage (stage IA or IIA) patients tend to have a relatively slow disease progression, while those diagnosed at later stages (stage IIB or above) often face a significantly shorter median survival, generally less than five years [14]. Early-stage diagnosis occurs in approximately 73% of MF cases [15].

1.1.4 Characteristics of pruritus in mycosis fungoides

Pruritus is one of the most frequently reported, long lasting, distressing, and challenging clinical symptoms for individuals with CTCL [16-18]. It impacts a substantial proportion of CTCL patients, with as many as 88% of all CTCL patients, 61% of those with MF, and 94% of Sézary syndrome cases experiencing this symptom [17, 19]. The intensity of pruritus is documented to increase with the stage of CTCL. Pruritus is a pivotal factor that significantly affects patient's physical and emotional well-being, sleep quality, functional

aspects, and general health status, leading to a marked reduction in their quality of life (QoL) in CTCL patients [18, 20-22].

Despite pruritus being widespread and intense among CTCL patients, it often remains resistant to conventional anti-pruritic treatments like topical steroids or oral antihistamines [19, 23, 24]. This lack of effective treatment options is largely attributed to the limited knowledge of the mechanisms underlying itch in CTCL.

1.2 Mast cells and MRGPRX2 may play important roles in itch

1.2.1 Mast cell maturation and distribution

Mast cells (MCs) originate from hematopoietic progenitor cells and undergo a gradual maturation process once they reach their target tissues [25, 26]. They are primarily situated in areas that interface with the external environment, such as the skin, airways, and gastrointestinal tract [27]. Due to this strategic location, MCs are among the first responders to invading pathogens or antigens [27].

1.2.2 The function of mast cell in itch

MCs, which are pivotal in both innate and adaptive immunity [27], defend against a variety of threats including parasites, bacteria, and toxins [25, 28, 29]. When activated, MCs release a range of inflammatory mediators associated with allergies, such as histamines, tryptase, cytokines, and growth factors, from their secretory granules [27]. This enables them to carry out their biological functions, including their contribution to the initiation and progression of pruritus [30].

Research has linked MCs to the development of various inflammatory and immune-related diseases that involve itch. For instance, patients with allergic contact dermatitis [31], atopic dermatitis, and psoriasis exhibit a significant increase in MCs in the lesional skin compared to healthy individuals [32]. Individuals suffering from chronic spontaneous urticaria also exhibit a significant elevation in MC numbers in both affected and unaffected skin [33, 34], and the manifestations and symptoms of this condition are related to MC activation and release of associated mediators [33]. Moreover, as compared to non-lesional skin, there is a higher MC count in lesional skin of individuals with prurigo nodularis

[35] and in those with recessive dystrophic epidermolysis bullosa, a condition where intense itch is prevalent, noting increased MC infiltration and degranulation [36].

MCs intricately modulate the sensation of itch through both histamine-dependent and -independent pathways [37]. Histamine, a crucial pruritogenic mediator released by MCs, interacts with a range of receptors from H1R to H4R, with particular emphasis on H1R and H4R in the realm of pruritus [37]. When histamine binds to the H1 receptor, it triggers the opening of the transient receptor potential V1 channel on sensory neurons, initiating nerve impulses that result in the sensation of itch [38]. Although the role of the H4 receptor in itch is not completely understood, it is also thought to be connected to transient receptor potential V1 activation, as suggested by animal studies [38].

Tryptase, a protease released by MCs, possesses the capability to induce itch by activating proteinase-activated receptor-2 both in patients with atopic dermatitis [39] and in mouse models [40]. Furthermore, MCs may contribute to the initiation of itch by producing Interleukin-31 (IL-31) in pruritus-associated diseases, such as recessive dystrophic epidermolysis bullosa [36]. Various substances, including drugs, neuropeptides, and host defense peptides, can directly stimulate MCs via the Mas-related G protein-coupled receptor X2 (MRGPRX2), leading to itch [41-43].

1.2.3 Mast cell activation via MRGPRX2

Mas-related G protein-coupled receptors (MRGPRs), which are a diverse family of receptors with seven-transmembrane domains, are primarily found in the sensory neurons of the dorsal root ganglia [44-46]. These receptors, also located on MCs [46], can be activated by a variety of substances, including synthetic compounds and neuropeptides [47]. The MRGPRX subset, particularly MRGPRX2, is highly expressed in humans and associated with the initiation of itch [45, 46].

MRGPRX2 is primarily located on skin MCs and plays a critical role in Immunoglobulin E (IgE)-independent MC activation [42]. It can prompt the release of MC granules when it binds to a range of external and internal substances, including neuropeptides, immune-related peptides, and certain drugs [48].

Studies have shown an increase in the number of MRGPRX2-expressing (MRGPRX2+) cells in skin affected by conditions such as mastocytosis, chronic urticaria, and chronic prurigo [49-52]. Additionally, higher MRGPRX2 mRNA levels have been observed in the itchy skin of individuals with dermatologic conditions like atopic dermatitis [53]. In allergic contact dermatitis, increased levels of the MRGPRX2 agonist PAMP1-20 have been found in the skin [31]. Similarly, we have shown that in chronic prurigo lesions, both cortistatin levels, a highly potent agonist of MRGPRX2, and the number of MRGPRX2+ cells are elevated compared to non-lesional skin [52]. Despite these findings, the role of MRGPRX2 in itch associated with CTCL is still to be fully determined and warrants further study.

1.3 Research questions addressed in this dissertation

In this dissertation, the objective was to characterize the pruritus experienced by CTCL patients, evaluate the impact of CTCL-related itch on QoL, explore the underlying mechanisms of this pruritus, and identify potential therapeutic targets. Here, I present the results of the published investigation [54] which examine the prevalence of MRGPRX2-positive cells in the skin of patients with MF and examines their association with itch severity, along with other clinical and laboratory findings.

2 Methods

2.1 Study participants

This dissertation enrolled ten individuals with MF, consisting of one female and nine males, with an average age of 66.2 years. Clinical data, blood samples and skin biopsies were collected from all MF patients (see details below). Additionally, eight healthy volunteers, three females and five males averaging 49.0 years old, provided general demographic data and skin biopsies to serve as a control group. These healthy volunteers had no history of dermatological diseases, allergies, tumors, autoimmune disorders, or thyroid diseases and served as the negative control group. Detailed demographic and baseline clinical characteristics are extensively described in Table 1 and elsewhere [55]. This dissertation received ethical approval from the Ethics Committee of Charité - Universitätsmedizin Berlin (EA4/124/10), and all participants provided written informed consent before participating in this research.

Table 1. Demographics and baseline characteristics in mycosis fungoides patients and healthy controls

Parameter	Patients with MF	Healthy controls	p-value
Male,n (%)[a]	9 (90.0%)	5 (62.5%)	p=0.27
Age (years), mean ± SD[b]	66.2 ± 9.8	49.0 ± 14.6	p=0.01
Tryptase (µg/l), mean ± SD[b]	5.95 ± 1.97	5.05 ± 1.27	p=0.29
IgE (kU/l), median (IQR)[b]	36.6 (18.4-225.8)	103.9 (25.4-250.0)	p=0.51
Substance P (pg/ml), median (IQR)[b]	329.7(261.1-423.3)	239.6(227.6-250)	p=0.10
MBP (ng/ml), median (IQR)[b]	353.5(281.2-446.2)	238.2(171-400.7)	p=0.27
IL31 (pg/ml), median (IQR)[b]	883.6(669.9-917.9)	799.3(701.5-977.6)	p=0.76
ECP (µg/l), median (IQR)[b]	12.1(10.8-17.2)	9.8(5-11.5)	p=0.07

Abbreviations: ECP, Eosinophilic cationic protein; IQR, Interquartile range; MBP, Major basic protein; MF, Mycosis fungoides; n, Number; SD, Standard deviation.

[a]Chi-square test was used for testing whether two independent unordered binary categorical variables are related to each other.

[b]Two- sample t test or Mann–Whitney U test was used for testing the differences between two independent categories of parametric and non-parametric variables, respectively.

Mean ± standard deviation (normally distributed data) and median (Interquartile range) (nonnormally distributed data) were shown for numerical variables. Number and percentages were shown for categorical variables. This table has been modified from Hu et al., 2023.

2.2 Clinical parameters

Clinical interviews and examinations were conducted during regular patient visits to collect clinical characteristics. General information such as age (in years), gender (female/male), body mass index (BMI, kg/m^2), body surface area (BSA, %), skin type (I-VI), and disease duration (in years), were recorded.

The Visual Analogue Scale (VAS) is a 10-centimeter rating scale used to evaluate the severity of different symptoms [56]. While originally designed to assess pain intensity, the VAS has found applications in assessing various symptoms, including pruritus, mood, appetite, asthma, dyspepsia, and ambulation [56-58]. In this dissertation, the VAS was used to measure the average level of itch intensity experienced over the past 24 hours, last week, and last month. Participants were instructed to indicate the severity of their symptoms on a scale ranging from 0 (indicating no itch) to 10 (representing the most severe imaginable itch).

Additionally, in this dissertation, the severity of the disease and QoL impairment were assessed using the VAS, with respondents rating their condition on a scale from 0 (not at all) to 10 (worst imaginable severity) and a Likert scale ranging from 0 (not at all) to 3 (extremely).

2.3 Validated questionnaires for the assessment of disease burden

2.3.1 Itch-specific Quality of Life (ItchyQoL) Questionnaire

The Itchy-specific Quality of Life (ItchyQoL) questionnaire, created by N.S. Desai et al. in 2008, serves as a comprehensive tool aimed at assessing the QoL disruptions caused by itch [59]. It encompasses three domains: symptoms, functional limitations, and emotions, with 27 pruritus-specific items measuring both frequency and bother [59]. Items related to frequency are rated on a scale from 1 (never) to 5 (all the time), while those related to bother assess the level of discomfort on a scale from 1 (not bothered) to 5 (severely bothered) [59]. The German version of the ItchyQoL questionnaire [60] was used in this dissertation.

2.3.2 Dermatological Life Quality Index (DLQI)

The Dermatological Life Quality Index (DLQI) is the first dermatological index designed to assess the impact of skin conditions on QoL [61]. It consists of ten questions addressing various aspects of daily functioning, including symptoms, feelings, daily activities, leisure, work or school, personal relationships, and treatment [62]. Scores on each question range from 0 (no impact) to 3 (significant impact), with total scores from 0 to 30 and higher scores signifying a more profound influence of the patient's skin condition on their QoL. This index is categorized into five levels of effect: 0-1(no effect), 2-5 (minor effect), 6-10 (moderate effect), 11-20 (considerable effect), and 21-30 (extremely substantial effect) [61, 62].

2.3.3 Modified Severity-Weighted Assessment Tool (mSWAT)

The Severity Weighted Assessment Tool (SWAT) [63] and its modified version (mSWAT) [64, 65] are the most widely used and clinically important tools for quantifying skin tumor spread in CTCL. The mSWAT was employed in this dissertation. The investigator assessed the separately affected BSA (%) percentage for patches, plaques, and tumors across 12 distinct body regions, using the patient's palm and fingers as a 1% BSA metric [63]. For patients with erythroderma, the affected BSA was assessed for patches and/or plaques [63]. The affected BSA percentages were multiplied by designated values for each lesion type (patch = 1, plaque = 2, tumor = 4) and summed to calculate the mSWAT score [63]. A single evaluator consistently determined these scores for the duration of this dissertation.

2.4 Non-validated questions for the assessment of disease burden

In addition to the validated questionnaires, some general inquiries were included in the assessments. These questions explored whether patients had experienced some specific symptoms such as itch, fatigue, fever, or insomnia during the course of the disease, with responses recorded as "yes" or "no."

2.5 Blood collection and mediators' assessment

Blood samples were collected through venipuncture in the morning, with both serum and EDTA plasma obtained. The levels of major basic protein (MyBioSource, MBS9308460),

eosinophilic cationic protein (MyBioSource, MBS700481), substance P (R&D systems, KGE007), and IL-31 (Cisbio, 62HIL31PEG) was carried out using commercially available ELISA kits, following the recommended protocols provided by the respective manufacturers. Additionally, the serum levels of total IgE and tryptase were measured at the centralized laboratory of Labor Berlin GmbH in Berlin, Germany.

2.6 Skin biopsy collection

Skin punch biopsies, each measuring 6mm in diameter, were obtained from lesional and non-lesional areas of ten MF patients. An additional biopsy from eight healthy controls was collected for subsequent histological examination. For patients with MF, biopsy distribution was as follows: five from the upper arm, three from the trunk, and two from the shoulder. In the case of healthy controls, seven biopsies were taken from the upper arm and one from the lower extremities.

2.7 Histological analysis

2.7.1 Tissue fixation and embedding

Following the collection of skin tissue samples, they were placed in containers suitable for fixation. The tissue samples were then submerged in a 10% formalin fixative solution and allowed to fix in formalin for a duration of 16 hours (overnight) to ensure proper fixation. Subsequently, the tissue samples were removed from the fixative and rinsed briefly with distilled water to remove any excess formalin. The samples were then transferred to an alcohol series for dehydration, which typically involved sequential immersion in increasing concentrations of ethanol (e.g., 70%, 95%, and 100% ethanol). This dehydration process aimed to remove water content and ensure proper preservation for embedding. The dehydrated tissue samples were then transferred to an embedding system (HistoCore Arcadia Embedding System, Leica Biosystems, Wetzlar, Germany) for paraffin embedding. The tissue samples were embedded in liquid paraffin wax using the embedding system, allowing the paraffin to infiltrate the tissue samples thoroughly to ensure complete impregnation.

After the paraffin embedding process, the tissue samples were removed from the embedding system. To prepare the embedded tissue block for sectioning, any excess paraffin

wax from the sides was trimmed to create a flat surface. Thin sections, with a thickness of 5 µm, were sliced from the paraffin-embedded tissue block using a microtome (Cryotome FSE from Shandon, Waltham, USA). The thin tissue sections were then immersed in a warm water bath to facilitate flattening and adherence to glass slides. Once retrieved, the sections were allowed to air dry completely before proceeding with the staining.

2.7.2 Immunohistochemistry

Immunohistochemistry for MRGPRX2 staining was conducted on samples from ten patients and eight healthy controls. The process began with two rounds of deparaffinization in xylene, with each round lasting for 10 minutes, to remove paraffin wax. This was followed by rehydration in graded ethanol, including 2 minutes in 100% Ethanol twice, 2 minutes in 96% Ethanol, and 2 minutes in 70% Ethanol. The slides were then rinsed with distilled water to eliminate any residual ethanol.

Subsequently, proteinase K pretreatment was executed according to the manufacturer's instructions for 10 minutes at room temperature. After the pretreatment, a protein block was applied at room temperature for 10 minutes. Following this, the sections were incubated at 4°C overnight with the primary anti-human MRGPRX2 monoclonal antibody (ab167125, Abcam) at a 1:1000 dilution. The next day, the slides were allowed to warm for 45 minutes at room temperature, after which they were rinsed with washing buffer (TBS) to remove any excess primary antibody. The following steps included a 0.3% H_2O_2 treatment at room temperature for 5 minutes, an incubation with polymer-HRP anti-mouse antibody (K400111-2, Dako) at room temperature for 30 minutes, and the application of AEC substrate chromogen for 20 minutes at room temperature, with intermediate washing steps. Subsequently, the slides were washed in TBS, counterstained with Mayer’s hematoxylin (ab128990, Abcam), and mounted using an aqueous mounting medium. The sections were then examined using a fluorescence microscope BZ-X800 (Keyence) for MRGPRX2 staining.

2.7.3 Immunofluorescence

For patients with MF, tissue sections from both lesional and non-lesional skin, were additionally subjected to double staining for MRGPRX2 using an immunofluorescence approach and the MC marker tryptase. Tryptase, a serine protease selectively expressed

by MCs in the skin, is therefore ideally suited for identifying skin MCs [66, 67]. The process began with preparing the sections, deparaffinizing and rehydrating them as described earlier. Proteinase K pretreatment was executed according to the manufacturer's instructions for 10 minutes at room temperature. Following a 10-minute protein block application at room temperature, the sections were incubated in a humid chamber with a mixture of anti-MRGPRX2 (ab167125, Abcam) and anti-tryptase (ab134931, Abcam) antibodies overnight at a recommended temperature of 4°C.

The next day, after warming the slides for 45 minutes, the tissue sections were washed with TBS three times, with each wash lasting for three minutes to remove any excess primary antibody. Next, the slides were incubated with Alexa Fluor® 594-AffiniPure goat anti-mouse IgG antibody (Jackson ImmunoResearch, 115-585-166) in TBS and along with 2% goat normal serum, for 30 minutes in the dark at room temperature. Subsequently, the sections were incubated with Alexa Fluor® 488-AffiniPure goat anti-rabbit IgG antibody (Jackson ImmunoResearch, 111-545-144), which was also diluted in TBS and 2% goat normal serum, for 30 minutes in the dark at room temperature. Ensuring that washing steps were conducted using TBS between each incubation. Finally, a preserving reagent containing 4',6-diamidinyl-2-phenylindole (DAPI, 00-4959-52; Invitrogen) was applied to mount the sections. Examination of the sections was performed using a fluorescence microscope BZ-X800 for MRGPRX2-tryptase double staining.

2.7.4 Quantitative histomorphometry

The assessment of immunostained sections was performed independently and in a blinded manner by two practiced investigators (Man Hu and Nian Liu). We manually counted the number of positive cells across a minimum of five horizontally adjacent high-power fields. For immunohistochemistry staining, a magnification of ×200 was utilized, covering an area of 0.25 mm^2, whereas for immunofluorescence double staining, a magnification of ×400 was employed, encompassing an area of 0.31 mm^2. The mean values were computed "per field" and subsequently converted to "per mm^2."

2.8 Single-cell RNA sequencing data analysis

To identify cell populations expressing MRGPRX2 in skin tissues from both MF patients and healthy donors, a single-cell RNA sequencing (scRNAseq) analysis was conducted.

The scRNAseq data for MF skin tissues were obtained from the GEO database under accession code GSE128531 [68], and the other scRNAseq dataset for healthy human skin was sourced from the Tabula Sapiens project on cellxgene [69]. Initially, raw gene counts were normalized to account for library size, resulting in counts per million. This was followed by a logarithmic transformation to the normalized counts. Dimensional reduction, cell clustering, and cell type annotation using known marker genes were then performed.

2.9 Statistical analysis

In this dissertation, statistical analysis was carried out using SciPy 1.8.0 in Python 3.9.12. Two-sample t-tests and Mann-Whitney U tests were applied to compare parametric and non-parametric variables, respectively, between two independent groups. For comparisons across three or more groups, one-way ANOVA with Tukey's post hoc test for parametric data, or Kruskal-Wallis with Dunn's post hoc test for non-parametric data, was utilized. For assessing differences between paired comparisons, such as between lesional and non-lesional skin samples, either a paired t-test was used for parametric variables, or the Wilcoxon signed-rank test was used for non-parametric variables. Spearman's rank correlation was used for analyzing variable correlations. A p-value of less than 0.05 was considered statistically significant. scRNAseq data was analyzed with Scanpy 1.9.3.

3 Results

3.1 The number of MRGPRX2-expressing cells is higher in lesional skin of MF patients compared to their non-lesional skin and skin of healthy individuals

The number of MRGPRX2-positive cells was determined by quantitative immunohischemistry (Fig. 1A). In individuals with MF, the number of MRGPRX2-positive cells was significantly increased in lesional skin compared to non-lesional skin (Wilcoxon signed-rank test, average: 15.12 versus 6.84 cells/mm², p=0.04, Fig. 1B). When comparing MRGPRX2-positive cell number between MF patients and healthy controls, a significantly higher number of MRGPRX2-positive cells in lesional skin than skin of healthy individuals was observed (Mann-Whitney U test, mean: 15.12 versus 5.51 cells/mm², p=0.04, Fig. 1B). Nonetheless, the difference between non-lesional skin of MF patients and healthy skin was not statistically significant (Mann-Whitney U test, 6.84 versus 5.51 cells/mm², p=0.56, Fig. 1B).

3.2 The number of mast cells in lesional skin of MF patients is slightly, but not significantly, higher than in non-lesional skin

The number of MCs was evaluated in MRGRPX2-tryptase immunofluorescence double staining. This evaluation revealed a numerical, but not statistically significant, increase in MCs in the lesional skin compared to non-lesional skin in patients with MF, with averages of 11.49 versus 7.94 cells/mm² (p=0.16).

3.3 The number of MRGPRX2+ cells and MCs correlate in lesional skin of MF patients

In the lesional skin of patients with MF, there is a significant positive correlation between the quantity of MRGPRX2-positive cells and MCs, as evidenced by a Spearman's rank correlation coefficient of 0.73 (p=0.02, Fig. 1C). Conversely, in the non-lesional skin of these patients, the correlation was not statistically significant, with a Spearman's rank correlation coefficient of 0.53 (p=0.11).

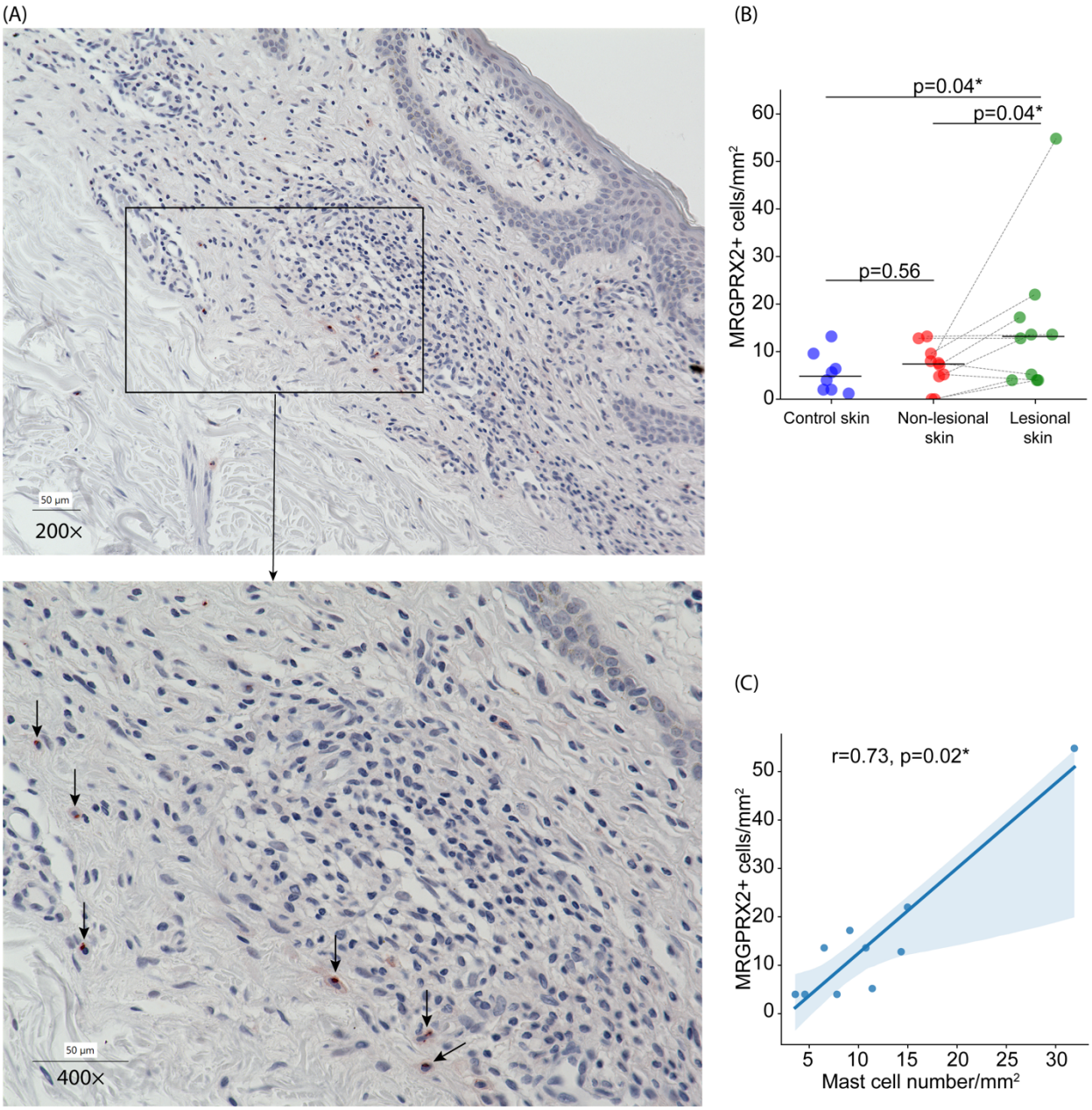

Figure 1. The number of MRGPRX2-expressing cells is increased in lesional skin of MF patients

(A) Immunohistochemical staining of MRGPRX2 in the lesional skin of patient with MF #6, ×200 (above) and ×400 (below) magnification (MRGPRX2+ cells are shown by black arrows). (B) The number of MRGPRX2-expressing cells in the lesional and non-lesional skin of MF patients and healthy control skin. (C) Correlation between the number of MRGPRX2-expressing cells and mast cells in the lesional skin of MF patients. Abbreviations: MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2. Mann–Whitney U test and Wilcoxon signed-rank test were used for testing the differences between unpaired data and paired data, respectively. Spearman rank correlation test was used for analyzing the correlation between two independent variables. $P < 0.05$ considered significant. *p<0.05. This figure is modified from Hu et al., 2023.

3.4 The number of MRGPRX2+ MCs is increased in lesional skin of MF patients

Double immunostaining for MRGPRX2 and the MC marker tryptase revealed the co-localization of MRGPRX2 and MCs (Fig. 2). While the prevalence of MRGPRX2-positive MCs in lesional skin of MF patients was higher compared to non-lesional skin, it did not significantly differ (average: 6.74 versus 4.04 cells/mm², p=0.11, Table 2). Notably, MRGPRX2+ MCs outnumbered MRGPRX2+ non-MCs in both lesional and non-lesional skin. Specifically, in lesional skin, MRGPRX2+ MCs were more prevalent than MRGPRX2+ non-MCs, though not significantly (average: 6.74 versus 3.45 cells/mm², p=0.09, Table 2). Contrastingly, in non-lesional skin, MRGPRX2+ MCs significantly outnumbered MRGPRX2+ non-MCs (average: 4.04 versus 2.44 cells/mm², p=0.01, Table 2).

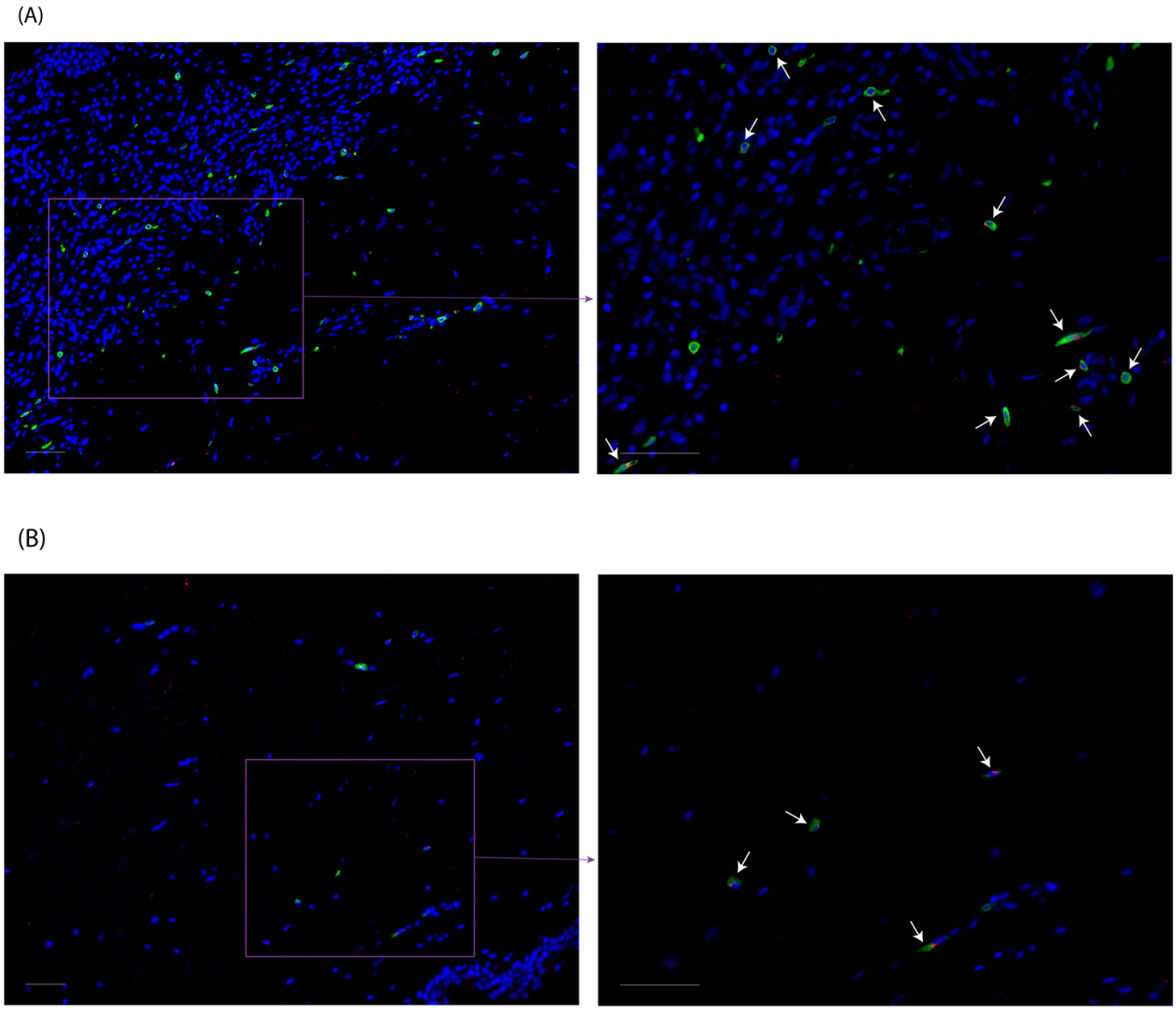

Figure 2. Co-localization of MRGPRX2 with the mast cell marker tryptase in MF patients

Immunofluorescence staining of lesional (A) and non-lesional (B) skin of the patient with MF #6 with anti-tryptase (green), anti-MRGPRX2 (red), and DAPI (blue). White arrows indicate some of tryptase-MRGPRX2 double-positive cells. ×200 (left) and ×400 (right) magnification. Bar = 50 μm. Abbreviations: DAPI, 4',6-diamidinyl-2-phenylindole; MF, Mycosis fngoides; MRGPRX2, Mas-related G protein-coupled receptor X2. This figure is modified from Hu et al., 2023.

Table 2. Co-localization of MRGPRX2 with the mast cell marker tryptase in patients with mycosis fungoides

Patients #	N of MCs, cells/mm^2	N of MRGPRX2+ cells, cells/mm^2	N of MRGPRX2+ MCs, cells/mm^2	N of MRGRPX2+ non-MC, cells/mm^2	Proportion of MRGPRX2+ MCs in MCs, %	Proportion of MRGPRX2+ MCs in MRGPRX2+ cells, %
P1L	7.81	2.28	1.95	0.33	25.00	85.71
P2L	9.11	10.09	5.86	4.23	64.29	58.06
P3L	10.74	9.11	4.23	4.88	39.39	46.43
P4L	4.56	5.21	2.60	2.60	57.14	50.00
P5L	11.39	7.16	2.28	4.88	20.00	31.82
P6L	31.90	34.51	27.99	6.51	87.76	81.13
P7L	14.97	8.79	7.49	1.30	50.00	85.19
P8L	14.32	13.67	7.81	5.86	54.55	57.14
P9L	6.51	6.84	4.88	1.95	75.00	71.43
P10L	3.58	4.23	2.28	1.95	63.64	53.85
Mean, lesional skin	**11.49**	**10.19**	**6.74**	**3.45**	**53.68**	**62.08**
P1NL	8.46	4.56	3.91	0.65	46.15	85.71
P2NL	6.51	4.56	2.60	1.95	40.00	57.14
P3NL	9.11	4.88	2.60	2.28	28.57	53.33
P4NL	5.21	3.91	2.60	1.30	50.00	66.67
P5NL	8.46	1.95	0.65	1.30	7.69	33.33
P6NL	11.72	14.97	10.42	4.56	88.89	69.57
P7NL	8.46	8.79	5.86	2.93	69.23	66.67
P8NL	6.51	3.91	2.60	1.30	40.00	66.67
P9NL	8.79	11.39	5.86	5.53	66.67	51.43
P10NL	6.18	5.86	3.26	2.60	52.63	55.56
Mean, non-lesional skin	**7.94**	**6.48**	**4.04**	**2.44**	**48.98**	**60.61**

Abbreviations: L, Lesional skin; MCs, Mast cells; MRGPRX2, Mas-related G protein-coupled receptor X2; N, Number; NL, Non-lesional skin.

Co-localization of MRGPRX2 with the mast cell marker tryptase was assessed by immunofluorescence. This table is modified from Hu et al., 2023.

3.5 No significant differences in the ratio of MRGPRX2+ MCs/MCs and MRGPRX2+ MCs/MRGPRX2+ cells between lesional and non-lesional skin of MF patients

The proportion of MRGPRX2+ MCs to the total MC count in lesional skin of MF patients was slightly higher than in their non-lesional skin, though this difference was not statistically significant (average: 53.68% versus 48.98%, p=0.38, Table 2). Similarly, the proportion of MRGPRX2+ MCs to the total number of MRGPRX2+ cells did not differ significantly between lesional and non-lesional skin (average: 62.08% versus 60.61%, p=0.86, Table 2).

3.6 Mast cells are the main cell population expressing MRGPRX2 in skin of MF patients and healthy subjects

Analysis of scRNAseq data from the skin of MF patients from publicly available sources [68] revealed that 5.5% of MCs express MRGPRX2, while lymphocytes and keratinocytes exhibited minimal expression at 0.01% for both. The vast majority of the MRGPRX2+ cells were MCs in MF (Fig. 3A-C). Moreover, in a distinct dataset derived from healthy skin tissues [69], MRGPRX2 expression was predominantly detected in 5.4% of MCs while T cells, macrophages, and stromal cells in the skin of healthy controls exhibited only very minimal expression at 0.19%, 0.12%, and 0.03%, respectively (Fig. 3D).

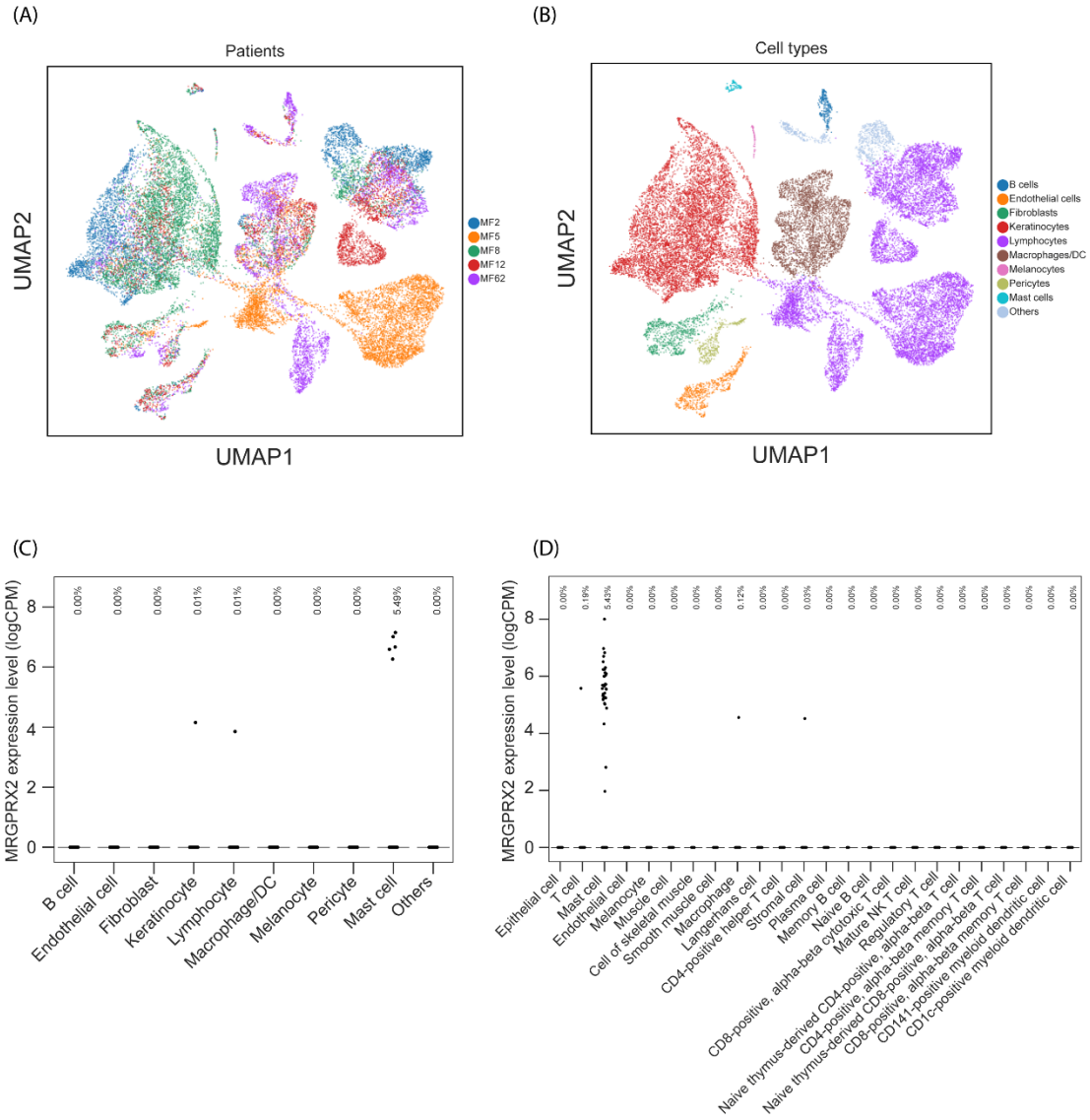

Figure 3. mRNA expression of MRGPRX2 in skin of MF patients and healthy skin tissues

UMAPs show the clusters of single cell transcriptomes of skin biopsies from five MF patients (A) and the cell types (B). Dot plot illustrates the mRNA expression levels of MRGPRX2 across different cell types in the skin of MF patients (C) and in healthy skin tissues from Tabula Sapiens (D). Each dot represents a single cell. Fractions of cells expressing MRGPRX2 are shown at the top of figure 3 (C) and (D). Abbreviations: MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2; UMAP, uniform manifold approximation and projection. This figure was modified from Hu et al., 2023.

3.7 High prevalence of pruritus in MF patients

In the population investigated for this thesis, pruritus was a common symptom among MF patients, with 70% reporting itch over the last 24 hours, week, and month. For those patients who reported experiencing pruritus, the mean itch intensity, as measured by VAS, was 1.9 ± 2.8 (mean ± SD) in the last 24 hours, 2.1 ± 3.1 over the last week, and 2.2 ± 3.1 over the last month.

3.8 Greater quality of life impairment in MF patients with pruritus

To assess the impact of pruritus on QoL, a VAS was used to evaluate QoL impairment and to compare the QoL in patients without pruritus against those with pruritus reported over the past week. MF patients with itch demonstrated significantly greater QoL impairment compared to those without (mean VAS: 2.64 versus 0, $p=0.02$). Additionally, itch intensity over the past 24 hours, week, and month showed very strong correlations with QoL impairment (Spearman's rank correlation: $r=0.93$, $p=0.0001$; $r=0.94$, $p<0.0001$; $r=0.95$, $p<0.0001$, respectively, Fig. 4). This itch intensity also strongly correlated with disease severity (Spearman's rank correlation: $r=0.83$, $p=0.003$; $r=0.86$, $p=0.001$; $r=0.90$, $p=0.0005$, respectively, Fig. 4).

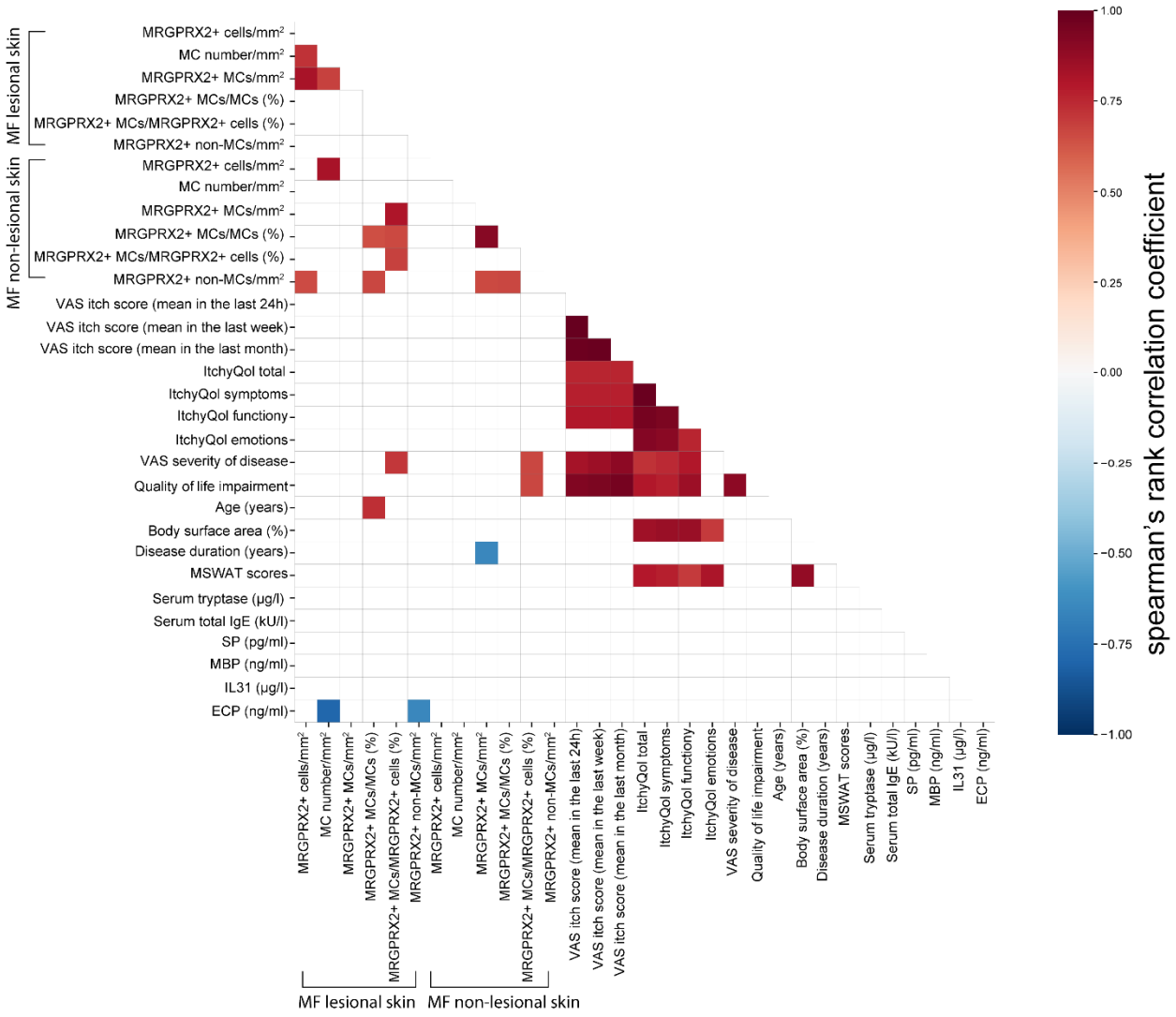

Figure 4. Spearman's rank correlation matrix between the variables in MF patients

The color of the cells represents correlation coefficients, indicating strength and direction of the correlations, ranging from red (positive correlations) to blue (negative correlations). The strength of the correlation is indicated in the color scale (at the right of the panel). Blank space indicates the correlation was not statistically significant (Spearman correlation with $P < 0.05$ considered significant). The values refer to valid data only (excluding missing data). Abbreviations: ECP, Eosinophilic cationic protein; IL31, Interlukin-31; ItchyQol: Itch-specific quality of life questionnaire; MBP, major basic protein; MC, Mast cell; MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2; MSWAT score, Modified severity-weighted assessment tool score; QOL, Quality of life; SP, Substance P; VAS, Visual analogue scale. This figure was modified from Hu et al., 2023.

3.9 The proportion of MRGPRX2+ MC/total MCs in non-lesional skin is higher in MF patients with itch than in those without itch

In comparison to MF patients without itch, those experiencing itch in the last week exhibited a significantly higher proportion of MRGPRX2+ MCs to the total MC count in non-lesional skin (mean: 59.08% vs. 25.42%, p=0.03). However, no significant difference of this proportion in lesional skin was observed between MF patients with and without pruritus in the last week. The quantities of MRGPRX2+ cells, MCs, MRGPRX2+ MCs, and the proportion of MRGPRX2+ MCs to MRGPRX2+ cells in both lesional and non-lesional skin did not significantly differ between MF patients with and without pruritus.

Furthermore, in the examined group of MF patients, no significant correlation was found between the itch intensity in the last 24 hours, last week, or last month and the counts or ratios of MRGPRX2+ cells, MCs, MRGPRX2+ MCs, MRGPRX2+ MCs/ MCs, or MRGPRX2+ MCs/ MRGPRX2+ cells in both lesional and non-lesional skin.

3.10 The ratio of MRGPRX2+ MCs/MRGPRX2+ cells correlated with the severity of disease in MF patients

A significant correlation was found between the proportion of MRGPRX2+ MCs to total MRGPRX2+ cells in both affected and unaffected skin and disease severity (Spearman's rank correlation coefficient of 0.71 and 0.67, with p-values of 0.02 and 0.03, respectively). No substantial differences were detected in gender distribution and serum levels of total IgE between MF patients and healthy individuals, though the latter group was significantly younger (as detailed in Table 1). The age of participants did not show a correlation with the number of MRGPRX2+ cells or MCs in the skin in both MF patients and healthy individuals. Additionally, the presence of MRGPRX2+ cells was not significantly linked to disease severity, duration, pruritus, QoL impairment, eosinophil counts, or the serum levels of total IgE, tryptase, eosinophilic cationic protein, IL-31, substance P, and major basic protein (Fig. 4).

4 Discussion

In my dissertation, I have identified a high prevalence of pruritus and its considerable impact on QoL in MF patients. Pruritus is one of the most severe and challenging symptoms in CTCL patients [70], affecting up to 88% of all CTCL patients [19], 61% of MF patients and 94% of Sézary syndrome patients [17]. The findings presented here are in line with these numbers, with a presence of pruritus in 70% of MF. In a study involving 414 MF patients, the average VAS values for the worst itch intensity ranged from 3.4 in early stages to 6.6 in late stages [17]. Here, lower VAS values have been detected for mean itch intensity within the last 24 hours (mean VAS: 1.9), week (2.1), and month (2.2). Although this variance could be due to the smaller sample size used in this dissertation, it is important to note that we have documented the VAS for the mean, and not the worst, itch. The intensity of the worst itch, as reported in the referenced literature, may be a more accurate and appropriate parameter than the mean itch intensity used here. The impact of pruritus on QoL in CTCL patients has been well-documented in previous research. Ottevanger et al. demonstrated in CTCL patients in different disease stages that itch is a significant symptom closely linked to reduced QoL [18, 71]. This dissertation reaffirms this correlation. The detrimental effect on QoL stems from the distressing nature of itch itself and the vicious cycle involving itch. Addressing QoL impairment is crucial in CTCL management, and effective itch control significantly improves treatment efficacy.

MCs have been implicated in the development of inflammatory diseases and pruritus [37, 72]. They are also recognized to have a pro-tumorigenic effect and are considered as prognostic markers and potential therapeutic targets in CTCL [73]. However, the relationship between MCs and CTCL-associated pruritus has not been established. In this dissertation, no significant differences in total IgE levels were observed between patients with MF and healthy controls. However, in another study of mine with a larger patient cohort [74], significantly higher serum total IgE levels were detected in MF patients compared to healthy controls, consistent with previous findings [75]. Despite this, the total IgE levels did not correlate with the itch intensity and were similarly elevated regardless of the presence of pruritus. Furthermore, there are no indications of a relevant IgE-mediated MC activation, i.e. no known specific IgE or auto-IgE antibodies, no wheal and flare-type reactions, and no relevant response to antihistamines in most MF patients. Therefore, a non-IgE and non-histaminergic mechanism underlying pruritus in MF is potentially more

important, and our data indicate that MRGPRX2-mediated MC activation is a likely candidate.

The results of my dissertation uncover a higher number of MRGPRX2-positive cells in lesional skin of individuals suffering from MF. In comparison to non-lesional areas, the increase of MRGPRX2-positive cells in lesional areas was slightly more pronounced in MF patients (with a 2.21-fold increase, mean: 15.12 versus 6.84 cells/mm^2) when compared to chronic prurigo, another skin diseases known to exhibit higher MRGPRX2 expressing (with a 1.50-fold increase, mean: 3.98 versus 2.66 cells/mm^2) [52]. However, it was notably lower than the increase observed in individuals with indolent systemic mastocytosis, a disease which is associated with a massive increase in skin MCs (with a 4.29-fold increase, median: 22.3 versus 5.2 cells/mm^2) [49].

Most MRGPRX2-positive cells in the skin of both healthy individuals and MF patients are MC, as indicated by immunofluorescence double stainings and the scRNAseq analysis. An increase in MRGPRX2-positive cells and MRGPRX2-positive MCs was observed in lesional skin of MF patients, a finding that is consistent with other MC-related skin disorders like chronic prurigo [52], chronic spontaneous urticaria [51], and cutaneous mastocytosis [50]. These findings may partially explain the observed correlation between MRGPRX2+ cells and MCs in the affected skin of MF patients in this dissertation.

While it is well-established that the majority of MRGPRX2-positive cells in most MF patients are MCs, it's worth noting that other cell types expressing MRGPRX2 may also be of significance, such as sensory neurons [76], keratinocytes [76], basophils, and eosinophils [77]. In indolent systemic mastocytosis, MRGPRX2-positive cells correlated with eosinophil counts [49]. Furthermore, microarray data suggests that T cells, known contributors to MF pathogenesis, might express MRGPRX2, though this is yet to be confirmed by real-time PCR [78]. scRNAseq data analysis in this dissertation revealed only a minimal presence of T cells expressing MRGPRX2 mRNA.

While we have identified an increase in MRGPRX2-positive cells, this did not correlate with the clinical or laboratory features in MF subjects. Similar findings were observed in patients with indolent systemic mastocytosis [49]. Nevertheless, the affirmative correlation between the proportion of MRGPRX2-positive MCs in lesional areas to total MRGPRX2-positive cell count and the severity of the disease underscores the potential

clinical significance of MRGPRX2-positive MCs in MF, necessitating further investigation in subsequent studies.

MRGPRX2 may influence the biology of connective tissue MCs, with the impact of MRGPRX2 ligands, which could account for MC activation and itch induction, potentially outweighing the number of MRGPRX2-positive cells. Notably, serum levels of substance P, a known MRGPRX2 agonist, exhibited a significant increase and showed a positive correlation with disease severity in MF patients [79]. Cortistatin (CST) has the capacity to trigger MC degranulation, and an elevated number of CST+ cells and CST+ MCs were found in lesional skin of chronic prurigo [52]. Additionally, CST expression was identified in lymphomas and lymphocytic leukemias [78].

Activation of MCs through MRGPRX2 and subsequent tryptase release has been linked to non-histaminergic pruritus, most likely by activating protease-activated receptor 2 on sensory nerves [31, 42], as observed in atopic dermatitis-related pruritus [39]. Another study of mine with a larger patient cohort demonstrated upregulated tryptase levels in MF patients with pruritus, but not in those without, and tryptase levels correlated with current itch intensity [74].

There are additional factors that may contribute to the absence of a connection between MRGPRX2 and the clinical aspects of MF. These could involve changes in expression due to receptor internalization or genetic variations [80, 81]. Furthermore, it's essential to consider that itch in MF patients might not only result from MRGPRX2 pathway activation, and thus other mechanisms should also be investigated.

IL-31, a cytokine closely linked to chronic pruritus in various diseases [82], is another mediator of interest in the context of MF-associated pruritus. While IL-31 can be produced by several cellular sources, including MCs and eosinophils [83, 84], it is thought to be primarily produced by activated Th2 cells [85]. Another study of mine found elevated serum IL-31 in pruritic MF patients, correlating with itch intensity [74]. This is in line with a previous report demonstrating that malignant CD4+ T cells obtained from CTCL patients can produce IL-31, with levels correlating with pruritus severity in advanced disease [86]. Moreover, elevated IL-31 levels were detected in the epidermis and dermal infiltrate of CTCL patients, with epidermal IL-31 levels showing correlation with itch severity [87], and improved pruritus correlated with reduction of IL-31 levels [88]. These findings indicate

IL-31 as a promising therapeutic target for CTCL-associated itch, particularly in advanced disease stages.

5 Conclusions

In conclusion, these findings underscore the critical need for pruritus assessment and management in CTCL patients. Treatment options for CTCL-associated pruritus are currently extremely limited, highlighting the urgent need for novel therapeutic targets. This dissertation emphasizes the necessity of investigating the roles and significance of MRGPRX2, its ligands, and MCs in MF. Future research should expand to larger patient cohorts and determine the levels of MRGPRX2 ligands in MF patient skin, providing a foundation for potential MRGPRX2-targeted treatments.

6 Limitation

This dissertation's findings are constrained by the small patient sample size. Although scRNAseq technology does have limitations, making it less effective in detecting genes with low expression, such as MRGPRX2, the diminished MRGPRX2 detection in scRNAseq does not introduce any bias when performing comparative analyses within the same dataset. Although healthy controls were significantly younger than MF patients, our analysis revealed no correlation between the age of participants and the quantities of MRGPRX2+ cells or MCs in the skin. Therefore, age does not introduce bias to our analysis. The final limitation of this dissertation is that the worst itch intensity seems to be a more suitable parameter for recording itch severity than the mean itch intensity, as described in the discussion section.

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Statutory Declaration

"I, **Man Hu**, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "**Characterization of the role and relevance of mas-related G protein-coupled receptor X2 expression in the skin of patients with mycosis fungoides**" (German translation: Charakterisierung der Rolle und Relevanz der Expression von mas-related G protein-coupled receptor X2 in der Haut von Patienten mit Mycosis fungoides), independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of your own contribution to the publications

Man Hu contributed the following to the below listed publications:

Hu M, Pyatilova P, Altrichter S, Sheng C, Liu N, Terhorst-Molawi D, Lohse K, Ginter K, Puhl V, Maurer M, Metz M and Kolkhir P. In the skin lesions of patients with mycosis fungoides, the number of MRGPRX2-expressing cells is increased and correlates with mast cell numbers. Front Immunol. 2023

Contribution (please set out in detail):

- First authorship
- Designed the project together with Dr. Martin Metz and Dr. Pavel Kolkhir
- Analyzed and interpreted the immunohistochemistry and immunofluorescence staining data
- Performed the immunofluorescence double staining in Figure 2
- Essential contribution to visualization of figure 1, 2 and 4
- Developed the initial draft of the manuscript
- Revised the previous versions of the manuscript by incorporating feedback from all coauthors with Dr. Martin Metz and Dr. Pavel Kolkhir.

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Signature, date and stamp of first supervising university professor / lecturer

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In the skin lesions of patients with mycosis fungoides, the number of MRGPRX2-expressing cells is increased and correlates with mast cell numbers

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Background: Mycosis fungoides (MF) is an indolent T-cell lymphoma that mainly affects the skin and presents with itch in more than half of the patients. Recently, the expression of Mas-related G protein-coupled receptor X2 (MRGPRX2), a receptor of mast cell (MC) responsible for the IgE-independent non-histaminergic itch, has been shown in lesional skin of patients with pruritic skin diseases, including chronic urticaria, prurigo, and mastocytosis. As of yet, limited knowledge exists regarding the MRGPRX2 expression in the skin of patients with MF.

Objectives: To investigate the number of MRGPRX2-expressing (MRGPRX2+) cells in the skin of patients with MF and its correlation with clinical and laboratory characteristics of the disease.

Methods: MRGPRX2 was analyzed in lesional and non-lesional skin of MF patients and healthy skin tissues by immunohistochemistry. Co-localization of MRGPRX2 with the MC marker tryptase was assessed by immunofluorescence. Public single-cell RNAseq data was reanalyzed to identify the MRGPRX2 expression on the distinct cell types.

Results: In lesional skin of MF patients, MRGPRX2+ cell number was higher than in non-lesional skin and healthy control skin (mean:15.12 vs. 6.84 vs. 5.51 cells/mm^2, p=0.04), and correlated with MC numbers (r=0.73, p=0.02). MC was the primary cell type expressing MRGPRX2 in MF patients. The ratio of MRGPRX2+ MCs to MRGPRX2+ cells in lesional and non-lesional skin correlated with the severity of disease (r=0.71, p=0.02 and r=0.67, p=0.03, respectively).

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Conclusions: Our findings point to the role of MRGPRX2 and MC in the pathogenesis of MF that should be investigated in further studies.

KEYWORDS

cutaneous T cell lymphoma, mycosis fungoides, pruritus, MRGPRX2, mast cells

Introduction

Mycosis fungoides (MF) is a type of peripheral non-Hodgkin T-cell lymphoma characterized by its predominant manifestation in the skin. It accounts for 60% of all cutaneous T-cell lymphomas (CTCL) and nearly 50% of all primary cutaneous lymphomas. The disease typically progresses through three stages: patch, plaque, and tumor stage (1). In patients diagnosed with MF, those in the early-stage (stage I or IIA) typically exhibit a relatively indolent disease course. However, individuals with advanced-stage MF (stage IIB or higher) have a considerably poorer prognosis, characterized by a median survival of less than 5 years (2).

MF significantly affects patients' quality of life (QoL), with itch being one of the most troublesome symptoms (3), occurring in up to 61% of MF patients (4). We have recently reported an association of chronic itch with increased disease severity, a more extensive involvement of the body surface area (BSA), and a pronounced impairment of QoL (5). The mechanisms for itch development in MF patients are not clear. Itch in many MF patients remains refractory to treatment including topical corticosteroids, ultraviolet light, and antihistamines (6).

Recently, Mas-related G protein-coupled receptor X2 (MRGRPX2) has been identified as a mast cell (MC) receptor responsible for IgE-independent MC activation and non-histaminergic itch (7). The MRGPRX2 can cause MCs to release their granules upon binding to a wide range of cationic substances, including neuropeptides, quorum sensing molecules from bacteria, venom peptides, host defense peptides, and FDA-approved drugs (8). Increased numbers of MRGPRX2-expressing (MRGPRX2+) cells have been reported in lesional skin of patients with various skin disorders including mastocytosis (9, 10), chronic urticaria (11) and chronic prurigo (12). As of yet, almost nothing is known about the role and relevance of MRGPRX2 in patients with MF. Here, we investigated the number of MRGPRX2+ cells in the skin of patients with MF and its correlation with itch and other clinical and laboratory characteristics.

Abbreviations: BSA, Body surface area; CST, Cortistatin; CTCL, Cutaneous T-cell lymphomas; DLQI, Dermatological life quality index; IL-31, Interleukin-31; ItchyQol, Itch-specific quality of life questionnaire; MC, Mast cell; MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2; mSWAT, Modified severity-weighted assessment tool; QoL, Quality of life; scRNAseq, Single-cell RNA sequencing; UMAP, Uniform manifold approximation and projection; VAS, Visual analogue scale.

Methods

Study population

The study was reviewed and approved by the Ethics Committee of the Charité - Universitätsmedizin Berlin (EA4/124/10). The patients/participants provided their written informed consent to participate in this study.

Ten patients with MF (1 female and 9 male, mean age 66.2 years) and 8 healthy controls (3 female and 5 male, mean age: 49.0 years) were included in the study. The patients' demographic characteristics have been collected and reported in eTable 1 and elsewhere (5). Briefly, disease severity was assessed by visual analogue scale (VAS), BSA scales, modified severity-weighted assessment tool (mSWAT), and a Likert scale (0–3). Average itch in the last 24h, last week and last month was also assessed by VAS, ranging from 0 (no itch) to 10 (worst imaginable itch). Pruritus-specific and skin-specific QoL impairments were assessed using Itch-specific quality of life questionnaire (ItchyQol) and the Dermatological Life Quality Index (DLQI), respectively. Patients were also asked about the presence of fatigue, fever, or insomnia. Laboratory parameters, including total serum IgE and serum tryptase levels, were determined at a central laboratory (Labor Berlin GmbH, Berlin, Germany). Eosinophilic cationic protein, major basic protein, IL-31, and substance P were measured using commercial ELISA kits, following the manufacturer's instructions. Two 6mm diameter skin punch biopsies were taken from MF patients (one from lesional skin and one from non-lesional skin), and one biopsy was taken from healthy controls for histological analysis. In 10 MF patients and 8 healthy controls, biopsies were taken from the upper arm (n=5 and n=7), the trunk (n=3 and n=0), the shoulder (n=2 and n=0) and the lower extremities (n=0 and n=1).

Histological analysis

MRGPRX2 staining by immunohistochemistry (10 patients and 8 healthy controls) and co-localization of MRGPRX2 with the MC marker tryptase by immunofluorescence (10 patients) were performed as described before (12) (the detailed description is provided in the Supplementary File online). Examination of the sections (MRGPRX2 staining and MRGPRX2-tryptase double staining) were carried out using a fluorescence microscope (BZ-X800; Keyence, Itasca, USA). The evaluation of the immunostained sections was done independently and blindly by two experienced

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investigators. The positive cells were manually counted in at least five horizontally adjacent high-power fields in the upper papillary dermis (for immunohistochemistry staining, ×200, 0.25 mm^2; for immunofluorescence double staining, ×400, 0.31 mm^2). Mean values per field were calculated and further converted to "per mm^2".

Eosinophil staining was performed as previously described (5). Briefly, wax blocks were cut into 5 µm sections and stained with Giemsa (Merck KG, Darmstadt, Germany) for histology. Two independent and blinded trained investigators counted eosinophils in five or more horizontally adjacent high-power fields (×400, 0.15 mm^2) in each three layers of papillary dermis, and the average cell numbers per horizontal layer were calculated.

Single-cell RNAseq data analysis

In order to determine the specific cell types expressing MRGPRX2, we conducted a single-cell analysis of MRGPRX2 expression using publicly available single-cell RNAseq (scRNAseq) data from MF skin tissues (13) and from healthy human skin (14). The scRNAseq data for MF skin tissues were downloaded from the GEO database (accession code: GSE128531), while the healthy human skin data were obtained from cellxgene (Tabula Sapiens). Preprocessing of the scRNAseq data involved normalizing the raw genes counts to the library size, resulting in counts per million. Subsequently, a log transformation was applied to the normalized data. Cell annotation was performed using unknown marker genes.

Statistical analysis

The statistical analysis was performed using the SciPy (version 1.8.0) in Python 3.9.12. Differences between two independent categories of parametric and non-parametric variables were evaluated using a two-sample t-test or a Mann-Whitney U test, respectively. Differences between three or more independent categories of parametric and non-parametric variables were evaluated using one-way analysis of variance (ANOVA), with a Tukey test used as a *post hoc* analysis, or the Kruskal-Wallis test, with a Dunn test used as a *post hoc* analysis, respectively. The differences between lesional and non-lesional skin biopsy samples were compared using paired t-test or the Wilcoxon signed-rank test for parametric and non-parametric variables, respectively. The correlation between variables was analyzed using Spearman's rank correlation. Statistical significance was set at $P < 0.05$. scRNAseq analysis was performed using Scanpy 1.9.3.

Results

In MF patients, the number of MRGPRX2+ cells in lesional skin was significantly higher compared to non-lesional skin and healthy skin (mean: 15.12 vs. 6.84 vs. 5.51 cells/mm^2, respectively, p=0.04) (Figures 1A, B). The number of MRGPRX2+ cells correlated with MC numbers in lesional (r=0.73, p=0.02) (Figure 1C) but not non-lesional skin of MF patients. Double staining for MRGPRX2 and tryptase indicated the co-localization of MRGPRX2 with MCs (Figure 2). The number of MRGPRX2+ MCs was higher as compared to MRGPRX2+ non-MCs in both lesional (mean: 6.74 vs. 3.45 cells/mm^2, p=0.09) and non-lesional skin (mean: 4.04 vs. 2.44 cells/mm^2, p=0.01). scRNAseq analysis of publicly available data from the skin of MF patients (13) and healthy skin (14) showed that 5.43-5.49% of MCs express MRGPRX2, whereas lymphocytes and keratinocytes showed minimal expression at 0.01% in the skin of MF patients (Figure 3). The ratio of MRGPRX2+ MCs to MRGPRX2+ cells in lesional and non-lesional skin correlated with the severity of the disease (r=0.71, p=0.02 and r=0.67, p=0.03, respectively).

MF patients and healthy controls did not differ in terms of gender and total serum IgE levels, although healthy controls were statistically significantly younger than MF patients (eTable 1). The age of patients and controls did not correlate with the number of MRGPRX2+ cells or with MC numbers in the skin. Lesional skin and non-lesional skin of MF patients did not significantly differ in numbers of MCs, eosinophils, MRGPRX2+ MCs, the ratio of MRGPRX2+ MCs to MCs, and the ratio of MRGPRX2+ MCs to MRGPRX2+ cells (eTable 2). The number of MRGPRX2+ cells did not correlate with disease severity, disease duration, pruritus, QoL impairment, eosinophil numbers, and serum levels of tryptase, total IgE, substance P, IL-31, eosinophilic cationic protein, and major basic protein (Figure 4).

Discussion

This study demonstrates a higher number of MRGPRX2+ cells in lesional skin of MF patients. The increase in lesional MRGPRX2+ cells as compared to non-lesional skin was slightly higher in MF (2.21 times higher, mean: 15.12 vs. 6.84 cells/mm^2) as compared to previously reported in chronic prurigo (1.50 times higher, mean: 3.98 vs. 2.66 cells/mm^2) (12) but lower than in indolent systemic mastocytosis (4.29 times higher, median: 22.3 vs. 5.2 cells/mm^2) (9). Increased numbers of MRGPRX2+ MCs were seen in lesional skin of patients with MC-driven disorders, such as chronic spontaneous urticaria (11), chronic prurigo (12) and cutaneous mastocytosis (10). In line with this, the immunofluorescence analysis and reanalysis of scRNAseq datasets provided further evidence supporting the predominant expression of MRGPRX2 on MCs in both healthy donor skin and MF patients' skin (13, 14). This finding offers a potential explanation for the positive correlation observed between the number of MRGPRX2+ cells and MCs in lesional skin of MF patients seen in our study.

Although in most MF patients the majority of MRGPRX2+ cells are MCs, other MRGPRX2-expressing cells might be relevant including sensory neurons (15), keratinocytes (15), basophils and eosinophils (16). In patients with indolent systemic mastocytosis, the number of MRGPRX2+ cells correlated with eosinophil number (9). However, we did not see such a correlation in MF patients, and eosinophils were rarely seen in skin samples. As shown by micro-array data, MRGPRX2 is expressed on T cells, which are pathogenic drivers in MF, although not confirmed by

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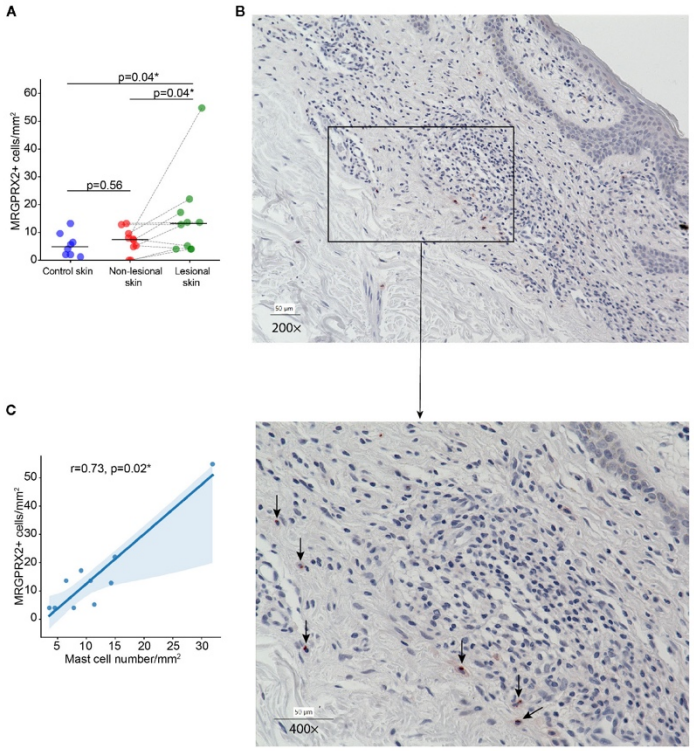

FIGURE 1
The number of MRGPRX2-expressing cells is increased in lesional skin of MF patients. **(A)** The number of MRGPRX2-expressing cells in lesional and non-lesional skin of MF patients and healthy control skin. **(B)** Immunohistochemical staining of MRGPRX2 in the lesional skin of patient with MF #6, ×200 (above) and ×400 (below) magnification (MRGPRX2+ cells are shown by black arrows). **(C)** Correlation between the number of MRGPRX2-expressing cells and mast cells in lesional skin of MF patients. MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2. Mann–Whitney U test and Wilcoxon signed-rank test were used for testing the differences between unpaired data and paired data, respectively. Spearman rank correlation test was used for analyzing the correlation between two independent variables. $P < 0.05$ considered significant. *p<0.05.

real-time PCR and further studies are needed (17). In our scRNAseq data analysis, we could see a small number of T cells expressing MRGPRX2 mRNA.

While the number of MRGPRX2+ cells was elevated, we did not observe any significant correlation with clinical or laboratory characteristics of MF in our patient's cohort. Similarly, the number of MRGPRX2+ cells did not correlate with the clinical and laboratory characteristics of patients with indolent systemic mastocytosis (9). However, the positive correlation between the ratio of lesional MRGPRX2+ MCs to MRGPRX2+ cells and disease severity points to the clinical relevance of MRGPRX2+ MCs in MF that should be investigated in further studies.

We have not determined skin levels of MRGPRX2 ligands that could account for MCs activation and itch induction. In this context, MRGPRX2 might reflect the development of connective tissue MCs and the number of MRGPRX2+ cells may be less important than the lesional presence of MRGPRX2 agonists, e.g. neuropeptides such as substance P and cortistatin (CST). The serum levels of substance P, an agonist of MRGPRX2, were significantly increased in MF patients and positively correlated with disease severity (18). CST can activate MCs for degranulation and increased numbers of CST-expressing cells and CST-expressing MCs were observed in lesions of chronic prurigo (12). Similarly, CST expression was found in lymphomas and lymphocytic leukemias (17).

Other factors can be responsible for the lack of association between MRGPRX2 and clinical features of MF including altered expression due to receptor internalization and/or genetic polymorphisms (19, 20). Lastly, itch in MF patients might not be dominantly triggered via MRGPRX2 pathway and other mechanisms, e.g. IgE-dependent MCs activation, should be ruled out (21).

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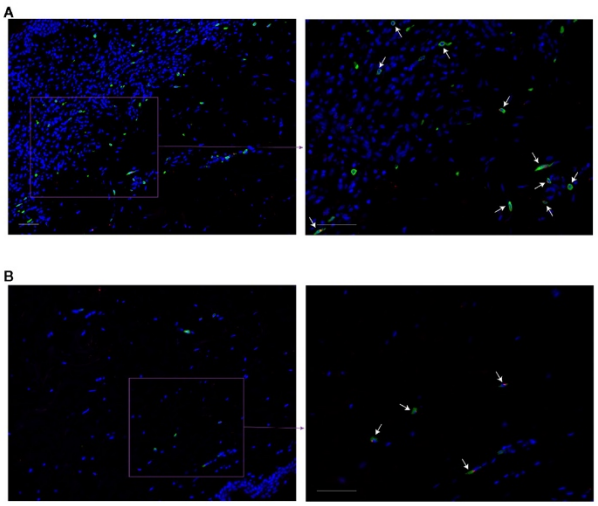

FIGURE 2
Co-localization of MRGPRX2 with the mast cell marker tryptase in MF patients. Immunofluorescence staining of lesional **(A)** and non-lesional **(B)** skin of the patient with MF #6 with anti-tryptase (green), anti-MRGPRX2 (red), and DAPI (blue). White arrows indicate some of tryptase-MRGPRX2 double-positive cells. ×200 (left) and ×400 (right) magnification. Bar = 50 μm. DAPI, 49-6-diamidino-2-phenylindole dihydrochloride; MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2.

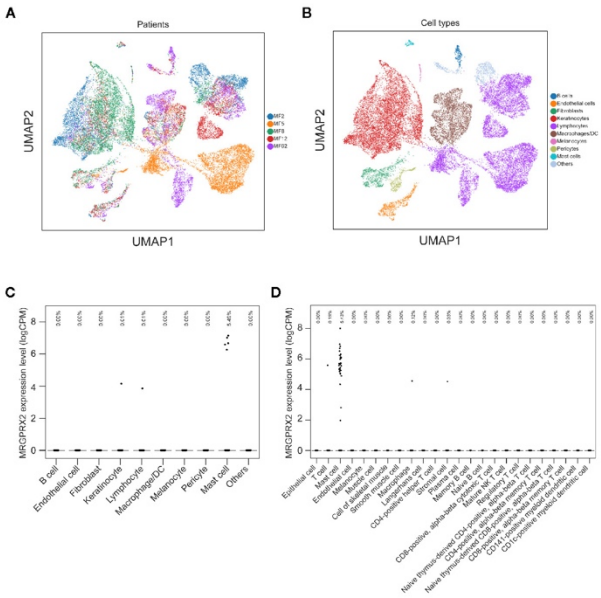

FIGURE 3
mRNA expression of MRGPRX2 in the skin of MF patients and the healthy skin tissues. UMAPs show the clusters of single cell transcriptomes of skin biopsies from five MF patients **(A)** and the cell types **(B)**. Dot plot illustrates the mRNA expression levels of MRGPRX2 across different cell types in the skin of MF patients **(C)** and in healthy skin tissues from Tabula Sapiens **(D)**. Each dot represents a single cell. Fractions of cells expressing MRGPRX2 are shown at the top of **(C, D)**. MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2; UMAP, uniform manifold approximation and projection.

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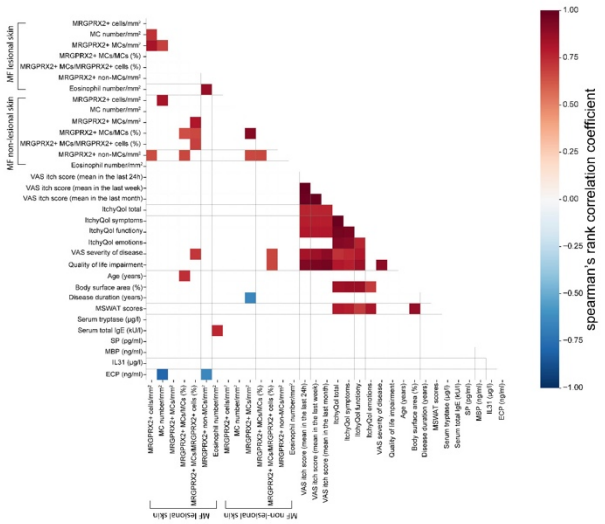

FIGURE 4
Spearman's rank correlation matrix between the variables in MF patients. The color of the cells represents correlation coefficients, indicating strength and direction of the correlations, ranging from red (positive correlations) to blue (negative correlations). The strength of the correlation is indicated in the color scale (at the right of the panel). Blank space indicates the correlation was not statistically significant (Spearman correlation with $P < 0.05$ considered significant). The values refer to valid data only (excluding missing data). ECP, Eosinophilic cationic protein; IL31, Interlukin-31; ItchyQol, Itch-specific quality of life questionnaire; MBP, major basic protein; MC, Mast cell; MF, Mycosis fungoides; MRGPRX2, Mas-related G protein-coupled receptor X2; MSWAT score, Modified severity-weighted assessment tool score; QOL, Quality of life; SP, Substance P; VAS, Visual analogue scale.

The results of our study are limited by the small number of patients. Despite the technology limitations of scRNAseq, which makes it less sensitive to lowly expressed genes like MRGPRX2, the lower detection of MRGPRX2 in scRNAseq does not introduce bias when conducting comparative analysis within the same dataset.

In conclusion, the role and relevance of MRGPRX2, its ligands and MCs in patients with MF need further investigation. Additional studies should include larger patient cohorts and determination of levels of MRGPRX2 ligands in the skin of patients with MF to provide a rationale for MRGPRX2-targeted treatments in this disease.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Charité - Universi-tätsmedizin Berlin (EA4/124/10). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PK, MMe, and MH designed the study and prepared the manuscript. PK, MMe, MH, PP, and CS analyzed and interpreted the data. PP, MH, and NL performed experiments. SA, DT-M, KL, KG, and VP collected the samples and clinical data. The study was supervised by PK and MMe. All coauthors critically revised and provided substantial input to the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SA has conducted studies for/was advisor for/was speaker for AstraZeneca, Allakos, ALK, Biocryst, CSLBehring, LeoPharma, Moxie, Novartis, Pharvaris, Sanofi, Takeda, Thermofisher. MaMe received honoraria advisory board, speaker from AbbVie, Amgen, AstraZeneca, argenx, Bayer, Beiersdorf, Celldex, Escient, Galderma, gsk, Jasper, Novartis, Pharvaris, Pfizer, Sanofi-Aventis, Tevapharm, ThirdHarmonicBio, Viforpharma, outside of submitted work. PK received honoraria advisory board, speaker from Novartis, Roche and ValenzaBio, outside of submitted work. Outside of this work, MMa is or recently was a speaker and/or advisor for and/or has received research funding from Astria, Allakos, Alnylam, Amgen, Aralez, ArgenX, AstraZeneca, BioCryst, Blueprint, Celldex, Centogene, CSL Behring, Dyax, FAES, Genentech, GIInnovation, GSK, Innate Pharma, Kalvista, Kyowa Kirin, Leo Pharma, Lilly, Menarini, Moxie, Novartis, Pfizer, Pharming, Pharvaris, Roche, Sanofi/Regeneron, Shire/Takeda, Third Harmonic Bio, UCB, and Uriach. DT-M has received research funds and/or was advisor for Celldex, Moxie, Novartis and Sanofi. Outside of this work, CS is an employee of the GV20 Therapeutics, which develops drugs and research models for profit.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1197821/full#supplementary-material

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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publication list

1. **Hu M**, Scheffel J, Frischbutter S, Steinert C, Reidel U, Spindler M, Przybyłowicz K, Hawro M, Maurer M, Metz M, Hawro T. Characterization of cells and mediators associated with pruritus in primary cutaneous T-cell lymphomas. Clin Exp Med. 2024 Jul 28;24(1):171. doi: 10.1007/s10238-024-01407-y. PMID: 39068637; PMCID: PMC11284195.

2. **Hu M**, Pyatilova P, Altrichter S, Sheng C, Liu N, Terhorst-Molawi D, Lohse K, Ginter K, Puhl V, Maurer M, Metz M* and Kolkhir P*. In the skin lesions of patients with mycosis fungoides, the number of MRGPRX2-expressing cells is increased and correlates with mast cell numbers. Front Immunol. 2023 Oct 30;14:1197821. doi: 10.3389/fimmu.2023.1197821. PMID: 38022672; PMCID: PMC10646224.

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