Increased expression of dermal LL-37 may trigger migration of CCR-7+regulatory T cells in extramammary Paget's disease

博士論文

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乳房外パジェット病における LL-37 を介した腫瘍免疫抑制システムの解明

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1. 要旨 Abstract

Background

Since extramammary Paget's disease (EMPD) is a skin adenocarcinoma of apocrine gland origin, the biological behavior of EMPD is similar to that of breast cancer. Invasive EMPD frequently metastasizes to lymph nodes, liver, lung and even brain. Therefore, an appropriate marker to distinguish in situ from dermal invasive EMPD in the early stage is needed. Since dermal LL-37 expression is augmented by stimulation with IL-17, and since Paget's cells produce CCL-20 to recruit Th17 in the lesional skin of EMPD, in this report, we hypothesized that LL-37 in the dermis is augmented in invasive EMPD.

Methods

We first employed immunohistochemical (IHC) staining of LL-37 in 15 cases of invasive and 11 cases non-invasive EMPD to evaluate the expression levels of LL-37 by digital microscope. Next, to evaluate the immunomodulatory effects of LL-37 on tumor-associated macrophages (TAMs) distributed in the dermis of the lesional skin of EMPD, we stimulated monocyte-derived M2 macrophages by LL-37 and measured the production of immunosuppressive chemokines using ELISA in vitro. Then, we further evaluated the serum level of these chemokine of EMPD patients.

Results

Quantitative analysis of IHC staining revealed that LL-37-expressing myeloid cells in dermis were significantly more in invasive EMPD than in non-invasive EMPD. Moreover, CD163+M2 macrophages produced CCL-19 and CCL-21 by LL-37 stimulation in vitro,

suggesting that these chemokines might be produced by CD163+TAMs to recruit CCR-7+regulatory T cells in the lesional skin of invasive EMPD. Indeed, immunofluorescence staining (IF) and IHC staining showed that substantial numbers of CD163+TAMs expressed CCL-19 and CCL-21 in the lesional skin of invasive EMPD. In addition, IHC staining revealed that CCR-7 positive cells (including regulatory T cells) were located within lymphatic vessels in the lesional skin of invasive EMPD. Furthermore, the serum levels of CCL-19 and CCL-21 were significantly increased in invasive EMPD patients compared to non-invasive EMPD patients.

Conclusion

The present study shows that TAMs around lymphatic vessels or small blood vessels produce LL-37 that stimulate the production of CCL-19 and CCL-21. These chemokines recruit CCR-7 +Tregs, which may play a role in the development of immunosuppressive microenvironment of invasive EMPD. These results suggest the potential of IL-17 blockade therapy to restore the immunosuppressive environment.

2. 略語一覧 List of abbreviations

- EMPD: extramammary Paget's disease
- CCL: chemokine (c-c motif) ligand
- CD: cluster of differentiation
- IL: interleukin
- MMP: matrix metalloproteinase
- RANK: receptor of activator nuclear factor kappa B
- RANKL: receptor of activator nuclear factor kappa B ligand
- RT-PCR: reverse transcription-polymerase chain reaction
- ELISA: enzyme-linked immunosorbent assay
- IF: immunofluorescence
- IHC: immunohistochemistry
- TAMs: tumor-associated macrophages
- TILs: tumor infiltrating lymphocytes
- PBMC: peripheral blood mononuclear cell
- M-CSF: macrophage colony-stimulating factor
- MDSCs: myeloid derived suppressor cells
- SCC: squamous cell carcinoma
- cSCC: cutaneous squamous cell carcinoma

3. 研究の背景 Research background

3.1. Extramammary Paget's disease

Extramammary Paget's disease (EMPD) is a rare malignant adenocarcinoma of apocrine gland origin that develops in scrotum, vulva, axilla, and penis [1]. Probably because the mammary gland that is an origin of breast cancer is an apocrine type of gland [2], the biological behavior of EMPD is similar to that of breast cancer, once it become invasive [3]. EMPD has a male predominance in Japan and Canada, and usually occurs in the sixth to eighth decades of life [4, 5]. The scrotum is the most common location in males, whereas the vulva is the most common in females [5]. Lesions clinically present as erythematous, well-demarcated plaques with or without erosion. Similar to breast cancer, invasive EMPD highly metastasizes to bone and draining lymph node [6]. In addition, since the treatment for metastatic EMPD is limited[7], appropriate markers to distinguish EMPD from invasive EMPD in the early stage is needed. Although there are several markers for the diagnosis of EMPD [3, 8-11], the useful marker for the detection of micro-invasion of Paget's cells in the dermis is lacking (Fig. 1).

3.2. LL-37 and IL-17 in psoriasis

Antimicrobial peptides and their precursor molecules form a central part of human and mammalian innate immunity, and play a critical role in mammalian innate immune defense against invasive bacterial infection [12]. LL-37 is a 37-residue, amphipathic, helical peptide found throughout the body that possesses a broad spectrum of antimicrobial activity [13, 14]. LL-37 binds to surface scavenger receptors expressed on various cells [e.g. myeloid dendritic cells (DCs), plasmacytoid DCs, macrophages to

recognize extracellular self-DNA [15-17]. CD163 that is a specific surface marker for macrophages is one of those scavenger receptors [18-20]. Psoriasis is one of the most common autoimmune-mediated skin diseases that manifests desquamative erythematous plaques. LL-37 is reported one of the auto-antigens that is significantly increased its expression on dendritic cells or keratinocytes in active psoriasis and responsible for the development of psoriasis [21, 22].Since the expression of LL-37 on myeloid cells in dermis is controlled by pro-inflammatory cytokines such as IL-17 and TNF-α [21], and since TNF-α/ IL-23/ IL-17 axis plays crucial roles in development of psoriasis, LL-37 is reported as one of the most important factors for the triggering of psoriasis [23].

3.3. The role of IL-17 in development of tumor

The significance of IL-17 in the development of various skin cancers has been reported recently [24-29] . For example, Wu *et al*. reported that IL-17 signaling in keratinocytes drives IL-17-dependent sustained activation of the TRAF4-ERK5 axis, leading to keratinocyte proliferation and tumor formation in cutaneous squamous cell carcinoma (cSCC) [24]. In another report, IL-17 and IL-22 increased the proliferation and migration of CAL27 squamous cell carcinoma (SCC) cell lines, suggesting the contribution of IL-17 to the progression of SCC [27]. Gasparoto *et al*. reported the significant co-relation of IL-17 expression in the precancerous microenvironment and the development of mouse cSCC [28]. Furthermore, we have reported that the lack of AhR signaling on keratinocytes in a 7,12-dimethylbenz[a]anthracene (DMBA)--induced SCC model significantly reduce the Th17-induced signaling, which resulted in the suppression of cSCC development [29]. The significance of IL-17 is reported not only in cSCC, but also in other types of skin

cancers. IL-17 produced by Th17 is positively associated with tumor angiogenesis such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) in melanoma [30]. Another report suggested that IL-17 enhances melanoma growth by the secretion of IL-6 from cancer stromal cells, which activates oncogenic STAT3 in melanoma cells [31]. More recently, we also reported the possible contribution of CCL-20/ IL-23/ IL-17 axis to the development of extramammary Paget's disease (EMPD) [26] (Fig.2). These reports suggested the significance of IL-17 in the carcinogenesis of cancers.

3.4. Immunological microenvironment of EMPD

Recently, anti-PD1 Abs-based immunotherapy for unresectable melanoma and non-melanoma skin cancer including EMPD has rapidly developed [32, 33]. As we described above, invasive EMPD highly metastasizes to bone and draining lymph nodes [3], but the treatment of metastatic EMPD is limited [7]. To apply immunotherapy for EMPD appropriately, the investigation of tumor microenvironment of EMPD is mandatory. From the above reasons, we have investigated the tumor microenvironment of EMPD, especially focusing on tumor-associated macrophages (TAMs) [8, 26, 34, 35]. We have demonstrated the following: 1) the number of CD163+TAMs was significantly higher in invasive EMPD than non-invasive EMPD [34]. 2) RANKL is expressed by Paget's cells in the lesional skin of EMPD and released to the tumor microenvironment in the presence of MMP-7[8]. Since RANKL can stimulates $CD163^{+}$macrophages to produce several unique cytokines including Treg-related chemokines (CCL-17) [26, 34], TAMs play crucial roles in maintaining the immunosuppressive tumor microenvironment of EMPD. 3) CCL-20 was expressed in the lesional skin of EMPD [25]. CCL-20 is a ligand for C-C

chemokine receptor, CCR-6, expressed on Th17. CCL-20 is produced by various cells including epidermal keratinocytes and tumor cells, leading to the recruitment of Th17 cells.

4. 研究の目的 Research purpose

The purpose of this study is to investigate the possible mechanisms for the development of the tumor immunosuppressive microenvironment in invasive EMPD by comparing the expression of LL-37 and several chemokines by TAMS among normal skin, the lesional skin of non-invasive EMPD and that of invasive EMPD.

5. 研究方法 Methods

5.1. Reagents

The following antibodies (Abs) were used for immunohistochemical and immunofluorescent staining: mouse monoclonal Abs against human LL-37 (Santa Cruz, San Diego, CA), human CCL-19 (LifeSpan BioScience, Seattle, WA), human Foxp3 (Abcam, Tokyo, Japan), and human CCL-21 (LifeSpan BioScience), rabbit polyclonal Abs against human CCR-7 (LifeSpan BioScience) and human CD163 (Enzo Life Science, Tokyo, Japan). We summarized the antibodies in Table 2.

5.2. Immunohistochemical staining, and immunofluorescence staining

Archived formalin-fixed paraffin-embedded skin specimens and cryosections from EMPD patients treated in the Department of Dermatology at Tohoku University Graduate School of Medicine, Sendai, Japan, were collected. All patients gave their informed consent. The study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine (2017-1-1045, 2017-1-425) and conducted according to the Declaration of Helsinki guidelines. The 10 non-invasive EMPD samples and 10 invasive EMPD samples were processed for single staining or double staining of Foxp3 and CCR-7. Briefly, formalin-fixed paraffin-embedded tissue samples were sectioned at 4 μm and deparaffinized. Antibody binding was demonstrated via alkaline phosphatase-conjugated anti-mouse Ig (Histofine SAB-AP(R) kit; Nichirei, Tokyo, Japan) for anti-Foxp-3 Ab or immunoglobulin from an unimmunized rabbit, and via peroxidase-conjugated anti-rabbit Ig (Histofine SAB-PO(M) kits; Nichirei) for the anti-CCR-7 Abs, or their isotype controls. Anti-Foxp3 Ab was developed with 3-Amino-9-ethylcarbazole (Wako Pure Chemical

Industries, Osaka, Japan), whereas the other mouse antibodies were visualized with 3, 3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries).

5.3. Quantitative analysis of immunohistochemical staining

To quantify the immunohistochemical staining of each sample, positive cells were counted using a BZ-X800 (KEYENCE, Tokyo, Japan). The percentage of IHC-positive cells per all tumour-infiltrating cells was counted automatically [36].

5.4. Culture of M2 macrophages from human peripheral blood monocytes

$CD14^+$monocytes were isolated from peripheral blood mononuclear cells from healthy donors using MACS beads (CD14 microbeads, Miltenyi Biotec Inc., Sunnyvale, CA) according to the manufacturer's protocol. $CD14^+$monocytes (2×10^5/ml) were cultured in complete medium containing 100 ng/ml of recombinant human M-CSF for 5 days, as previously reported (Fig. 3) [34]. On the fifth day, monocyte-derived macrophages were treated with or without LL-37 (20 ng/ml) for 48 hours and the culture supernatant was harvested. The study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine (2019-1-925) and conducted according to Declaration of Helsinki guidelines.

5.5. RNA extraction, RNA quality assessment, and reverse transcription and quantitative real-time PCR

Preserved formalin-fixed paraffin-embedded skin specimens and frozen sections were prepared from lesion tissues obtained from EMPD patients treated at the Department of

Dermatology, Tohoku University Hospital (Table 1). Informed consent was obtained from all patients. Immunohistochemical staining and immunofluorescence staining of frozen sections were performed. For frozen sections, each sample was frozen in optimal cutting temperature embedding medium, 6 μm sections were fixed with cold acetone for 30 minutes and blocked with IF buffer (PBS, 5% bovine serum albumin). Each section was then incubated with the relevant antibody. Slides were mounted on DAPI Fluoromount-G (Southern Biotech, Birmingham, AL) and photographed using a Zeiss LSM 700 microscope (Zeiss Japan, Tokyo, Japan) equipped with a SPOT digital camera.

5.6. Enzyme-linked immunosorbent assays (ELISAs)

The concentration of chemokine in the culture supernatant from M2 macrophages treated with various stimuli or serum levels of 15 cases of invasive EMPD (TNM staging: T1, T2) and 11 cases of non-invasive EMPD (TNM staging: Tis) (Table 1) patients were determined in using ELISAs for human CCL19/MIP-3 beta, human CCL-20 /MIP-3 alpha, human CCL-21/6Ckine, and human CCL22/MDC (Table 3) according to the manufacturer's protocols. The study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine (2019-1-306) and conducted according to Declaration of Helsinki guidelines.

5.7. Statistical analysis

For single comparisons between two groups, the Wilcoxon signed-rank test was used. The level of significance was set at $p<0.05$.

6. 研究結果 Results

6.1. Invasive extramammary Paget's disease increased LL-37 expression in dermal lesions

Since our group has already demonstrated that the lesional skin of EMPD is rich in CCL-20, IL-23, and IL-17 [26], we hypothesized that LL-37 may be expressed in the lesional skin of EMPD as seen in psoriasis which is well known to express both IL-17 and IL-23[37]. Since there is no significant expression of LL-37 in non-lesional skin (data not shown), we compared LL-37 expression between non-invasive EMPD and invasive EMPD. Immunohistochemistry staining of 15 cases of invasive EMPD (TNM staging: T1, T2) and 11 cases of non-invasive EMPD (TNM staging: Tis) were performed (Table 1). The numbers of LL-37+cells were significantly higher in the dermis of invasive EMPD (Fig.4) than in that of non-invasive EMPD (Fig. 4). The ratio of LL-37+cells to all infiltrating cells was significantly increased from 1.0% to 4.4% ($p<0.01$) (Fig. 4). Since LL-37 is controlled by IL-17 [21], we additionally evaluated the IL-17 expressing cells in invasive and non-invasive EMPD. The ratio of IL-17+cells to all infiltrating cells was significantly increased from 6.4% to 9.3% ($p<0.05$) (Fig. 5). Moreover, IHC staining of the serial sections revealed that the main population of LL-37-expressing cells was CD163+TAMs (Fig. 6).

6.2. LL-37 increased the production of CCL-19 and CCL-21 from monocyte-derived M2 macrophages in vitro.

We have already reported substantial numbers of CD163+TAMs were detected in the lesional skin of EMPD [8, 38]. In this study, we found CD163+TAMs expressed LL-37 (Fig. 6). LL-37 is known to induce a pro-inflammatory immunological microenvironment similar in psoriasis [39]. Since recent reports suggested the correlation between CCL-19 and/or CCL-21 with immunosuppressive microenvironment of skin cancer such as melanoma [40, 41], we next hypothesized that LL-37 might lead to the production of pro-inflammatory cytokines CCL-19, CCL-20 and CCL-21, as well as immunosuppressive chemokine CCL-22, from $CD163^{+}$M2 macrophages. To test this, the production of chemokines from $CD163^{+}$M2 macrophages was evaluated using M2 macrophages generated from peripheral blood mononuclear cells (PBMCs) of healthy donors (Fig. 3) [26, 34]. The production of CCL-19, CCL-20 and CCL-21 was significantly increased, whereas there was no increase of CCL-22 by LL-37 stimulation (Fig. 7).

6.3. CCL-19-producing cells, CCL-21-producing cells, and CCR-7-expressing cells in the lesional skin of EMPD.

LL-37 stimulation of CD163+M2 macrophages induced CCL-19, CCL-20 and CCL-21 production. In contrast to these in vitro findings, we found that CCL-20 was preferentially expressed in the epidermis of EMPD by IHC [26]. Thus, we examined the expression of CCL-19 and CCL-21 in EMPD tissues. There were substantial numbers of cells expressing CCL-19 (Fig. 8a) and CCL-21 (Fig. 8a) around not only small vessels, but also within lymphatic vessels in the dermis of invasive EMPD. Additionally, double immunofluorescence staining for CD163 and CCL-19, or CD163 and CCL-21 revealed that $CD163^{+}$TAMs expressed CCL-19 (Fig. 8b) and CCL-21 (Fig. 8b).

Since CCL-19 and CCL-21 are known to recruit CCR-7+cells, immunohistochemical staining for CCR-7 in the lesional skin of EMPD was performed. CCR-7+cells were detected in the lesional skin of EMPD even within lymphatic vessels (Fig. 9). As we previously reported, since the number of Foxp3+Tregs in invasive EMPD is significantly lower than that in non-invasive EMPD [42], we hypothesized that these CCR-7+cells contained Foxp3+Tregs. We employed immunohistochemical staining for Foxp3 (Fig. 9a) in the serial sections of the CCR-7 staining samples, which indicated that Foxp3+cells were also detected within lymphatic vessels. Furthermore, we performed double staining for CCR-7 and Foxp3 (Fig. 9b). CCR-7+positive cells detected within lymphatic vessels were Foxp3+Tregs (Fig.9c, 9d).

6.4. Serum levels of CCL-19 and CCL-21 were higher in patients with invasive EMPD than in those with non-invasive EMPD.

To further confirm the increased production of CCL-19 and CCL-21 in invasive EMPD, we examined the serum level of CCL-19 and CCL-21 of invasive and non-invasive EMPD patients. The results demonstrated that the serum levels of CCL-19 and CCL-21 were significantly higher in invasive EMPD than in non-invasive EMPD patients (Fig. 10). The clinical data of non-invasive and invasive EMPD patients are summarized in Table 1.

7. 考察 Discussion

The Immunopathological analysis of EMPD has been progressing in recent years. Indeed, several reports focused on the immunological background of EMPD, especially on tumor-infiltrating lymphocytes (TILs) [43-46] and their regulators, such as TAMs [8, 26, 34] and CD1a+CD207+Langerhans cells [35].

The IL-23/IL-17A pathway plays a critical role in the development of psoriasis [47, 48] and is a target for the treatment of psoriasis [47].

LL-37 is an autoantigen in psoriasis derived from keratinocytes that could trigger the initial activation of myeloid cells, as well as IL-17-producing cells, Th1 and Th22 cells [22]. Notably, LL-37 expression is reduced by IL-17 blockade, suggesting that IL-17 can augment the expression of LL-37 in the lesional skin of psoriasis [21]. In addition, previous reports revealed high serum levels of CCL-19 and CCL-21 in psoriasis patients [49, 50]. Like psoriasis, we have demonstrated that the IL-23/IL-17A pathway was activated in the lesional skin of EMPD, in which Paget's cells produce CCL-20 as well as IL-23 leading to the accumulation of Th17 cells [26].

As a previous report suggested, LL-37 can stimulate myeloid cells including macrophages [16]. TAMs are found in various skin cancers, including EMPD, where they maintain the immunosuppressive microenvironment together with other immune cells (e.g. Tregs, Th2 cells, Th17 cells, myeloid-derived suppressor cells (MDSCs)) [38]. The main population of TAMs in EMPD is CD163+M2 macrophages, which surround venules and/or arterioles

and lymphatic vessels in the superficial dermis [34, 42]. The present study revealed increased expression of LL-37 in dermal macrophages when Paget's cells infiltrated into the dermis. In addition, it also demonstrated in vitro that LL-37 stimulated the production of CCL-19 and CCL-21 by CD163+M2 macrophages. These results suggested the lesional skin of invasive EMPD recruited CCR-7+cells [51, 52]. Indeed, CCR-7+Foxp3+Tregs were found around and within lymphatic vessels. These Tregs may contribute to the immunosuppressive microenvironment of invasive EMPD in addition maintaining RANKL expression by Paget's cells as shown in mammary tumors [53].

In a series of our studies, we could demonstrate the activation of the IL-23/IL-17A pathway. It is well known that the pro-inflammatory cytokine IL-17 family has been associated with poor prognosis in breast cancer. Interestingly, both EMPD and breast cancer are characterized with frequent bone metastasis. IL-17 might be a culprit for bone metastasis by its stimulatory activity for osteoclastogenesis by upregulating RANKL on osteoclast-supporting mesenchymal cells [54].

Our studies had several limitation. We could not determine the source of IL-17 in the lesional skin of invasive EMPD. Since IL-17 is produced by neutrophils, innate-lymphoid cell 3, and gamma-delta T cells other than Th17 cells [55-57], further investigation is necessary to identify the source of IL-17.

Next, we have not directly demonstrated that IL-17 stimulates LL-37 production by macrophages. In the lesional skin of psoriasis or hidradenitis suppurativa, however, it has

been demonstrated that the expression level of LL-37 positively correlates with IL-17 expression and is significantly decreased by IL-17 blockade [21, 22, 58, 59].

Finally, we have not clearly demonstrated the mechanism by which increased production of CCL-19 or CCL-21 induces the migration of CCR-7+Tregs into lymph vessels. In allergic contact dermatitis, it is well known that CCL-19 or CCL-21 promotes the migration of CCR-7+dendritic cells to regional lymph nodes [60]. In that scenario, the surface-immobilized form of the chemokine CCL-21 and the heparan sulfate-anchoring ligand of the CCR-7, cause random movement of DCs because it triggers integrin-mediated adhesion. Upon direct contact with CCL-21, DCs truncate the anchoring residues of CCL-21, thereby releasing it from the solid phase. Soluble CCL-19 and CCL-21, which lacks anchoring residues, form soluble gradients. Adhesive random migration and directional steering cooperate to produce dynamic movement into secondary lymphoid organs [61]. The similar mechanism may occur in invasive EMPD.

In summary, the present study suggests that TAMs around lymphatic vessels or small vessels produce CCL-19 and CCL-21 by LL-37 stimulation leading to the recruitment of CCR-7+Tregs that might promote lymph node metastases or remote metastases of Paget's cells in invasive EMPD. The LL-37 expression might be augmented by IL-17 produced by Th17, which is recruited by CCL-20 derived from Paget's cells. (Fig.11). Anti-IL-17 antibodies, such as ixelizumab and secukinumab, are currently used clinically for the treatment of psoriasis, and they decrease the expression of LL-37 in the lesional skin of psoriasis [21]. The present study suggests the possible therapeutic effects of anti-IL-17

antibodies for the prevention of lymph node metastases or remote metastases of invasive EMPD.

8. 結論 Conclusion

The present study shows that TAMs around lymphatic vessels or small blood vessels produce LL-37 that stimulate the production of CCL-19 and CCL-21. These chemokines recruit CCR-7 +Tregs, which may play a role in the development of immunosuppressive microenvironment of invasive EMPD. These results suggest the potential of IL-17 blockade therapy to restore the immunosuppressive environment.

9. 利益相反 Conflict of interest

Regarding this research, there are no companies or corporations or profit-making organizations with conflicting interests to report.

10. 謝辞 Acknowledgements

本研究は平成 28 年より東北大学大学院医学系研究科神経・感覚器病態学講座皮膚科学教室にて行わせていただきました。

東北大皮膚科の藤村卓講師には指導教官として貴重な時間を費やしていただき多大な御助言と御指導を賜りましたことを深く感謝し、御礼申し上げます。

同教室の相場節也教授、神林由美先生、佐藤遥太先生、谷田佳世先生をはじめとする同教室の先輩方、医局員、大学院生、技術補佐員の方々にも多くの御支援と励ましをいただきましたことをここに深謝いたします。

Since 2016, this research has been carried out at the Department of Dermatology, Tohoku University Graduate School of Medicine.

I would like to express my deep gratitude and thanks to Dr. Taku Fujimura of the Tohoku University Dermatologist for spending valuable time as an instructor and for giving me a great deal of advice and guidance.

Prof. Setsuya Aiba, Dr. Yumi Kamibayashi, Dr. Yota Sato, Dr. Kayo Tanida, and other senior members of the classroom, medical staff, graduate students, and technical assistants provided their support and encouragement for which I would like to express my deep gratitude.

11. 参考文献 References

1. Heymann, W.R., Extramammary Paget's disease. Clin Dermatol, 1993. 11(1): p. 83-7.
2. Khan, Y.S., A. Farhana, and H. Sajjad, Anatomy, Thorax, Mammary Gland, in StatPearls [Internet]. 2019, StatPearls Publishing.
3. Shiomi, T., et al., Clinicopathological study of invasive extramammary Paget's disease: subgroup comparison according to invasion depth. J Eur Acad Dermatol Venereol, 2013. 27(5): p. 589-92.
4. Ghazawi, F.M., et al., Trends in incidence of cutaneous malignant melanoma in Canada: 1992-2010 versus 2011-2015. J Am Acad Dermatol, 2019. 80(4): p. 1157-1159.
5. Ghazawi, F.M., et al., Demographic and clinical characteristics of extramammary Paget's disease patients in Japan from 2000 to 2019. J Eur Acad Dermatol Venereol, 2020.
6. Maeda, T., et al., Extramammary Paget's disease patient-derived xenografts harboring ERBB2 S310F mutation show sensitivity to HER2-targeted therapies. Oncogene, 2020. 39(36): p. 5867-5875.
7. Tanese, K., et al., Updates on the Systemic Treatment of Advanced Non-melanoma Skin Cancer. Front Med (Lausanne), 2019. 6: p. 160.
8. Kambayashi, Y., et al., The Possible Interaction between Receptor Activator of Nuclear Factor Kappa-B Ligand Expressed by Extramammary Paget Cells and its Ligand on Dermal Macrophages. J Invest Dermatol, 2015. 135(10): p. 2547-2550.

9. Nowak, M.A., et al., Perianal Paget's disease: Distinguishing primary and secondary lesions using ummunohistochemical studies including gross cystic disease fluid protein-15 and cytokeratin 20 expression. Archives of pathology & laboratory medicine, 1998. 122(12): p. 1077.
10. Ohnishi, T. and S. Watanabe, The use of cytokeratins 7 and 20 in the diagnosis of primary and secondary extramammary Paget's disease. Br J Dermatol, 2000. 142(2): p. 243-7.
11. Fujimura, T., et al., RANKL expression is a useful marker for differentiation of pagetoid squamous cell carcinoma in situ from extramammary Paget disease. J Cutan Pathol, 2016. 43(9): p. 772-5.
12. Durr, U.H., U.S. Sudheendra, and A. Ramamoorthy, LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochim Biophys Acta, 2006. 1758(9): p. 1408-25.
13. Hancock, R.E., Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis, 2001. 1(3): p. 156-64.
14. Wiesner, J. and A. Vilcinskas, Antimicrobial peptides: the ancient arm of the human immune system. Virulence, 2010. 1(5): p. 440-64.
15. Burton, M.F. and P.G. Steel, The chemistry and biology of LL-37. Nat Prod Rep, 2009. 26(12): p. 1572-84.
16. Takahashi, T., et al., Cathelicidin promotes inflammation by enabling binding of self-RNA to cell surface scavenger receptors. Sci Rep, 2018. 8(1): p. 4032.
17. Chamilos, G., et al., Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL-37. Blood, 2012. 120(18): p. 3699-

707.

18. Fabriek, B.O., C.D. Dijkstra, and T.K. van den Berg, The macrophage scavenger receptor CD163. Immunobiology, 2005. 210(2-4): p. 153-60.

19. Lau, S.K., P.G. Chu, and L.M. Weiss, CD163A specific marker of macrophages in paraffin-embedded tissue samples. American journal of clinical pathology, 2004. 122(5): p. 794-801.

20. Van Gorp, H., P.L. Delputte, and H.J. Nauwynck, Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. Mol Immunol, 2010. 47(7-8): p. 1650-60.

21. Fuentes-Duculan, J., et al., Autoantigens ADAMTSL 5 and LL 37 are significantly upregulated in active Psoriasis and localized with keratinocytes, dendritic cells and other leukocytes. Experimental dermatology, 2017. 26(11): p. 1075-1082.

22. Lande, R., et al., The antimicrobial peptide LL-37 is a T-cell autoantigen in psoriasis. Nat Commun, 2014. 5(1): p. 5621.

23. Furue, M. and T. Kadono, The contribution of IL-17 to the development of autoimmunity in psoriasis. Innate Immun, 2019. 25(6): p. 337-343.

24. Wu, L., et al., A novel IL-17 signaling pathway controlling keratinocyte proliferation and tumorigenesis via the TRAF4-ERK5 axis. J Exp Med, 2015. 212(10): p. 1571-87.

25. Chen, C. and F.H. Gao, Th17 Cells Paradoxical Roles in Melanoma and Potential Application in Immunotherapy. Front Immunol, 2019. 10: p. 187.

26. Sato, Y., et al., Malassezia-derived aryl hydrocarbon receptor ligands enhance the

CCL-20/Th17/soluble CD163 pathogenic axis in extra-mammary Paget's disease. Exp Dermatol, 2019. 28(8): p. 933-939.

27. Nardinocchi, L., et al., Interleukin-17 and interleukin-22 promote tumor progression in human nonmelanoma skin cancer. Eur J Immunol, 2015. 45(3): p. 922-31.

28. Gasparoto, T.H., et al., Inflammatory events during murine squamous cell carcinoma development. J Inflamm (Lond), 2012. 9(1): p. 46.

29. Sato, Y., et al., Possible Roles of Proinflammatory Signaling in Keratinocytes Through Aryl Hydrocarbon Receptor Ligands for the Development of Squamous Cell Carcinoma. Front Immunol, 2020. 11: p. 534323.

30. Muranski, P., et al., Th17 cells are long lived and retain a stem cell-like molecular signature. Immunity, 2011. 35(6): p. 972-85.

31. Wang, L., et al., IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. Journal of Experimental Medicine, 2009. 206(7): p. 1457-1464.

32. Fujimura, T., et al., Treatment of Advanced Melanoma: Past, Present and Future. Life (Basel), 2020. 10(9): p. 208.

33. Ishii, M., et al., Anti-PD-1 antibody therapy for epithelial skin malignancies: An investigator-initiated, open-label, single-arm, multicenter, phase II clinical trial (NMSC-PD1 Study). Medicine (Baltimore), 2020. 99(44): p. e22913.

34. Fujimura, T., et al., Receptor Activator of NF-kappaB Ligand Promotes the Production of CCL17 from RANK+M2 Macrophages. J Invest Dermatol, 2015. 135(11): p. 2884-2887.

35. Fujimura, T., et al., Possible mechanisms of the crosstalk between Langerhans cells and regulatory T cells in extramammary Paget disease by receptor activator of nuclear factor kappa B (RANK) ligand/RANK pathways. Br J Dermatol, 2017. 176(2): p. 387-394.

36. Fujimura, T., et al., A novel technique to diagnose non-melanoma skin cancer by thermal conductivity measurements: Correlations with cancer stromal factors. Exp Dermatol, 2019. 28(9): p. 1029-1035.

37. Liu, T., et al., The IL-23/IL-17 Pathway in Inflammatory Skin Diseases: From Bench to Bedside. Front Immunol, 2020. 11: p. 594735.

38. Fujimura, T., et al., Tumor-Associated Macrophages: Therapeutic Targets for Skin Cancer. Front Oncol, 2018. 8: p. 3.

39. Li, N., et al., Alarmin function of cathelicidin antimicrobial peptide LL-37 through IL-36γ induction in human epidermal keratinocytes. The Journal of Immunology, 2014. 193(10): p. 5140-5148.

40. Cristiani, C.M., et al., Accumulation of Circulating CCR-7(+) Natural Killer Cells Marks Melanoma Evolution and Reveals a CCL19-Dependent Metastatic Pathway. Cancer Immunol Res, 2019. 7(5): p. 841-852.

41. Fankhauser, M., et al., Tumor lymphangiogenesis promotes T cell infiltration and potentiates immunotherapy in melanoma. Sci Transl Med, 2017. 9(407).

42. Fujimura, T., et al., Comparison of Foxp3+regulatory T cells and CD163+macrophages in invasive and non-invasive extramammary Paget's disease. Acta Derm Venereol, 2012. 92(6): p. 625-8.

43. Iga, N., et al., Accumulation of exhausted CD8+T cells in extramammary Paget's

disease. PLoS One, 2019. 14(1): p. e0211135.

44. Karpathiou, G., et al., Expression of CD3, PD-L1 and CTLA-4 in mammary and extra-mammary Paget disease. Cancer Immunol Immunother, 2018. 67(8): p. 1297-1303.

45. Mai, R., et al., Transcriptome analyses reveal FOXA1 dysregulation in mammary and extramammary Paget's disease. Hum Pathol, 2018. 77: p. 152-158.

46. Fletcher, J. and M. Haniffa, Mechanisms of immune evasion in extramammary Paget disease. Br J Dermatol, 2017. 176(2): p. 293-294.

47. Brembilla, N.C., L. Senra, and W.H. Boehncke, The IL-17 Family of Cytokines in Psoriasis: IL-17A and Beyond. Front Immunol, 2018. 9: p. 1682.

48. Dainichi, T. and K. Kabashima, Interaction of Psoriasis and Bullous Diseases. Front Med (Lausanne), 2018. 5: p. 222.

49. Pickens, S.R., et al., Characterization of CCL-19 and CCL-21 in rheumatoid arthritis. Arthritis Rheum, 2011. 63(4): p. 914-22.

50. Bosè, F., et al., Inhibition of CCR-7/CCL-19 axis in lesional skin is a critical event for clinical remission induced by TNF blockade in patients with psoriasis. The American Journal of Pathology, 2013. 183(2): p. 413-421.

51. Picchio, M.C., et al., CXCL13 is highly produced by Sezary cells and enhances their migratory ability via a synergistic mechanism involving CCL-19 and CCL-21 chemokines. Cancer Res, 2008. 68(17): p. 7137-46.

52. Basile, J., et al., Chemokine receptor expression in non-melanoma skin cancer. J Cutan Pathol, 2008. 35(7): p. 623-9.

53. Tan, W., et al., Tumour-infiltrating regulatory T cells stimulate mammary cancer

metastasis through RANKL-RANK signalling. Nature, 2011. 470(7335): p. 548-53.

54. Sato, K., et al., Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med, 2006. 203(12): p. 2673-82.

55. Zeng, B., et al., ILC3 function as a double-edged sword in inflammatory bowel diseases. Cell Death Dis, 2019. 10(4): p. 315.

56. Hu, S., et al., IL-17 Production of Neutrophils Enhances Antibacteria Ability but Promotes Arthritis Development During Mycobacterium tuberculosis Infection. EBioMedicine, 2017. 23: p. 88-99.

57. Roark, C.L., et al., gammadelta T cells: an important source of IL-17. Curr Opin Immunol, 2008. 20(3): p. 353-7.

58. Peric, M., et al., IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. J Immunol, 2008. 181(12): p. 8504-12.

59. Thomi, R., et al., Elevated levels of the antimicrobial peptide LL-37 in hidradenitis suppurativa are associated with a Th1/Th17 immune response. Exp Dermatol, 2018. 27(2): p. 172-177.

60. Worbs, T. and R. Forster, A key role for CCR-7 in establishing central and peripheral tolerance. Trends Immunol, 2007. 28(6): p. 274-80.

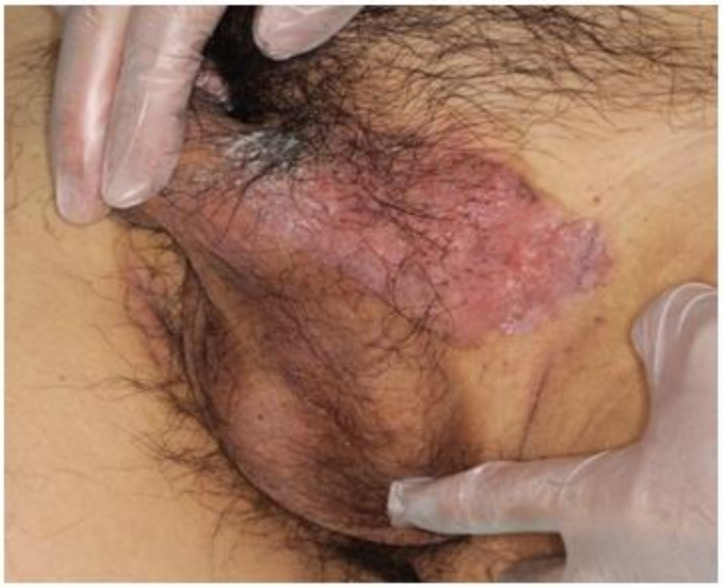
61. Schumann, K., et al., Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. Immunity, 2010. 32(5): p. 703-13.

12. 図説と図 Figure legends

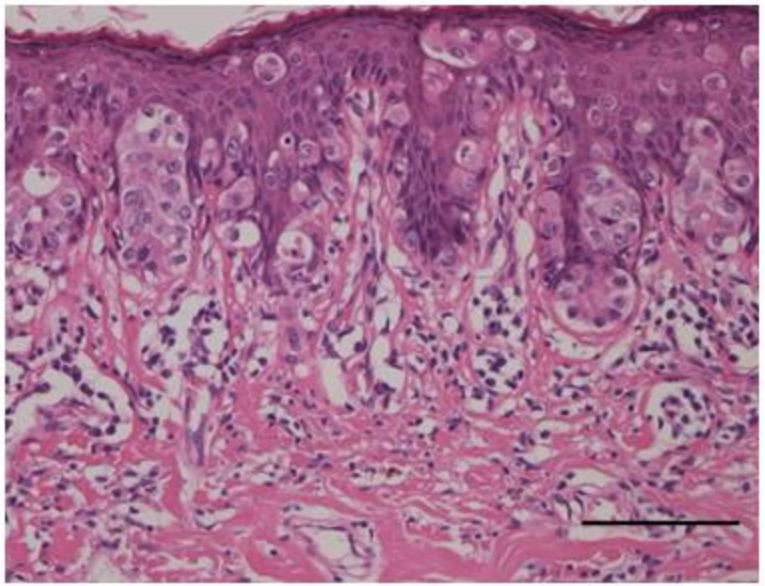
12.1. Fig. 1. Clinical and histological features of EMPD.

Clinical picture of extramammary Paget's disease (a). Histological findings of invasive extramammary Paget's disease from skin: rounded cells that were devoid of intracellular bridges and large nucleus (b). Scale bars, 200 μm.

a

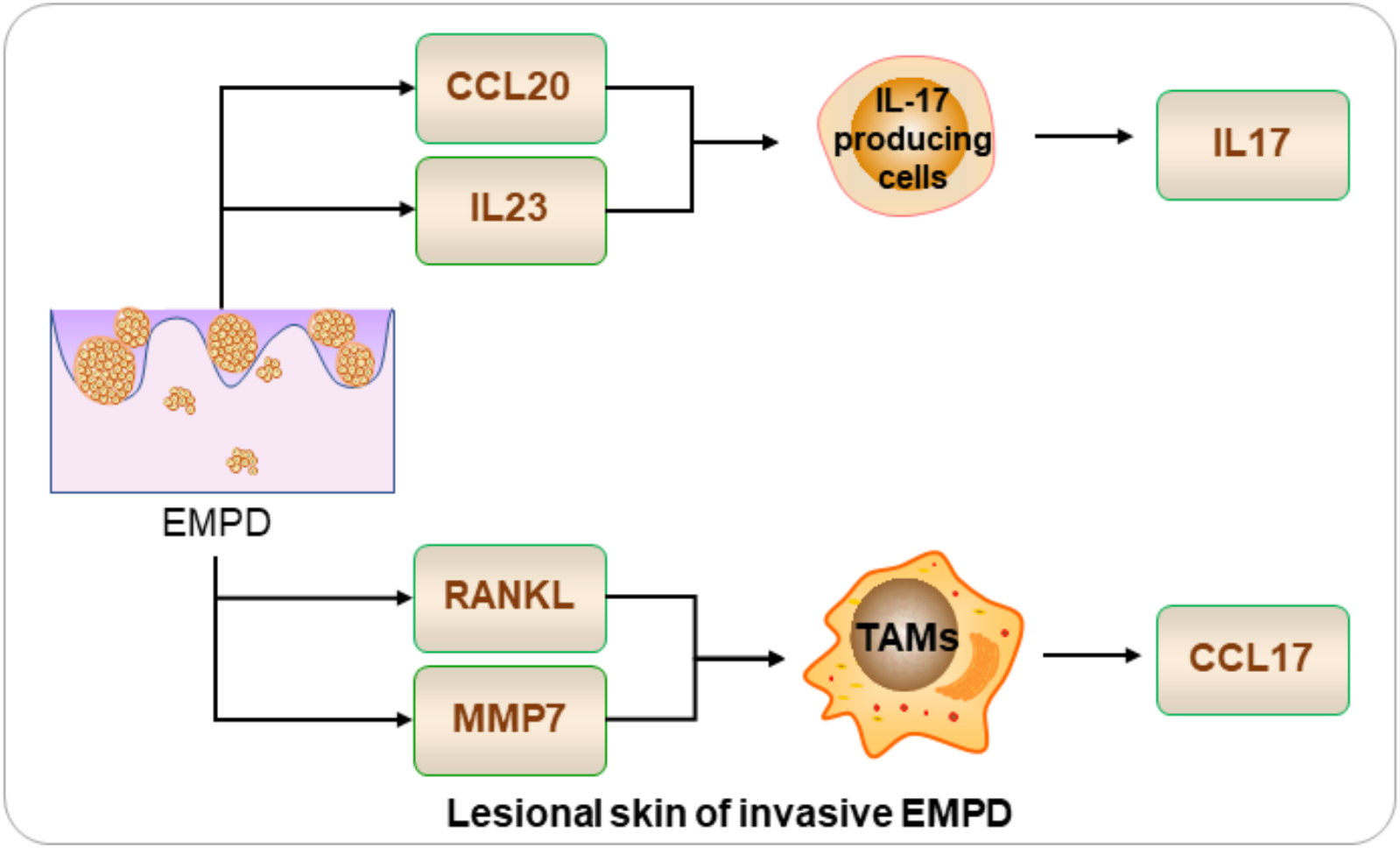


b



12.2. Fig.2. Th17-related signals in the tumor microenvironment of EMPD.

The CCL-20 / IL-23/ IL-17 axis and RANKL /MMP-7/ CCL-17 axis in the development of extramammary Paget's disease.



Kambayashi et al 2015, J Invest Dermatol

Fujimura et al 2015, J Invest Dermatol

Sato et al 2015 Exp Dermatol

12.3. Fig. 3 The induction of M2 macrophages from PBMC.

CD14 +monocytes were isolated from peripheral blood mononuclear cells of healthy donors using MACS magnetic beads (CD14 microbeads, Miltenyi Biotec Inc., Sunnyvale, California), and cultured in complete medium containing 100ng/ml of recombinant human M-CSF for 5 days. Then, monocyte-derived macrophages were treated with or without 20ng/ml LL-37 for 48 hours and the culture supernatant was collected. The culture supernatants was measured by ELISA.

PBMC

MACS® $CD14^+$

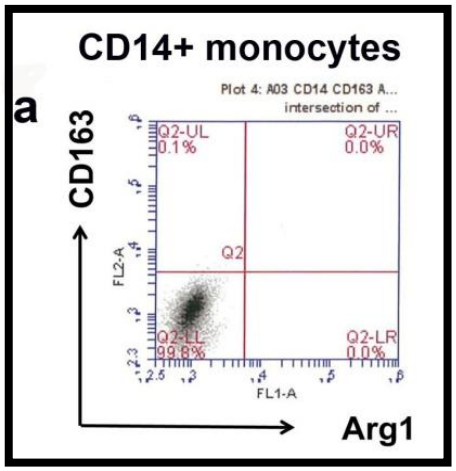
$CD14^+$ cell + M-CSF (100ng/ml)

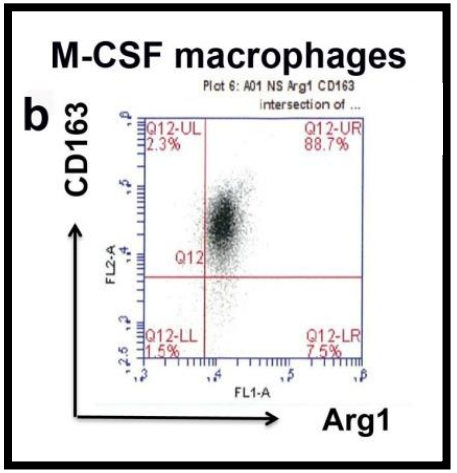
5 days

LL-37 (20ng/ml)

48h

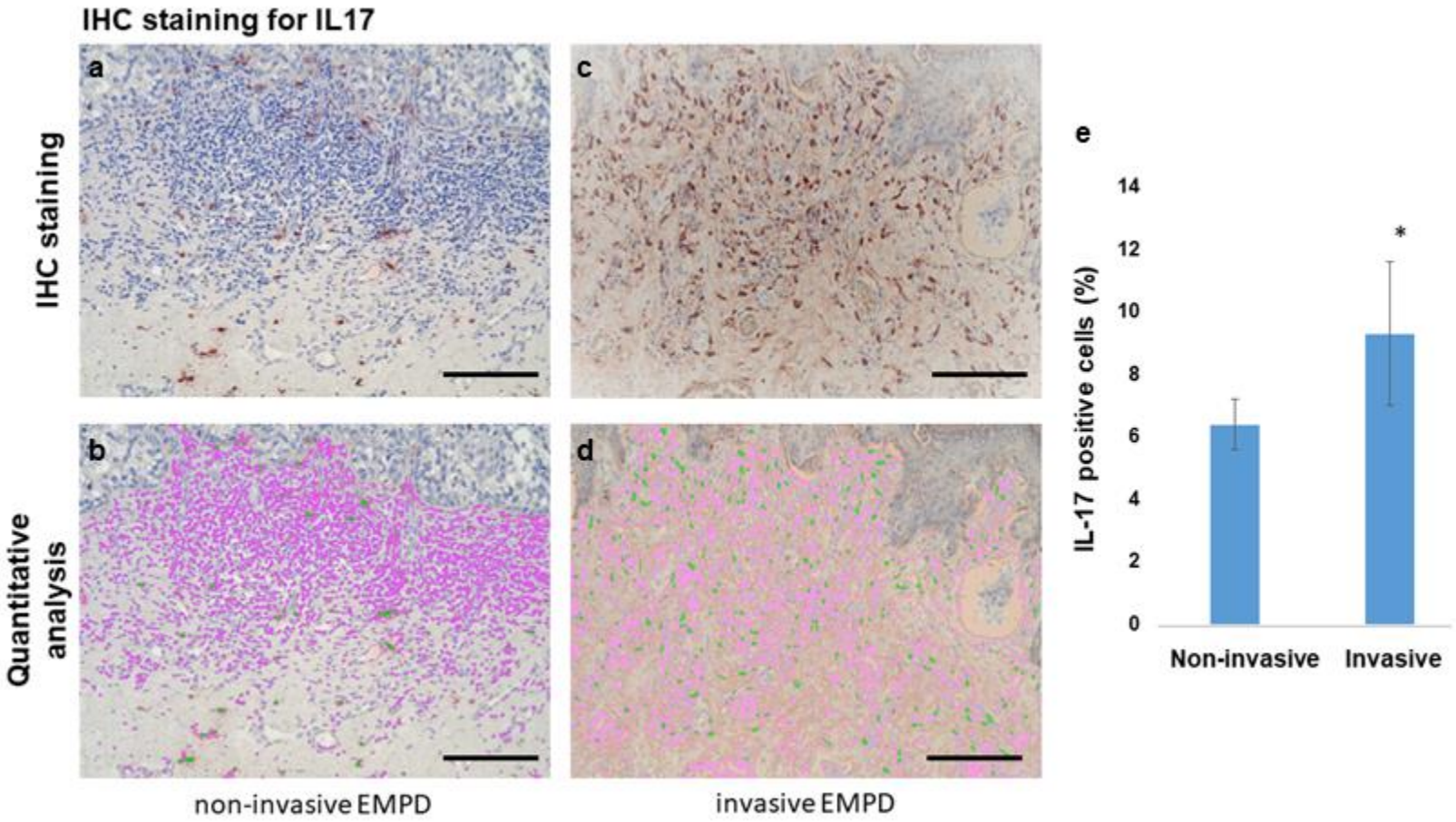
ELISA





12.4. Fig. 4. LL-37 expression cells increased in the dermal lesions of invasive EMPD.

Sections of invasive EMPD and non-invasive EMPD lesions were deparaffinized and stained using anti-LL-37 (a, c) and the number of LL-37-positive cells per all tumor-infiltrating cells in different sections of invasive EMPD and non-invasive EMPD were automatically counted by BZ-X800. Representative specimens from analyses of 10 cases of EMPD are shown (b, d). The ratio of LL-37 positive cells to all tumor-infiltrating lymphocytes was significantly increased in invasive EMPD (e).Scale bars, 100 μm (a, b), 50μm (c, d).

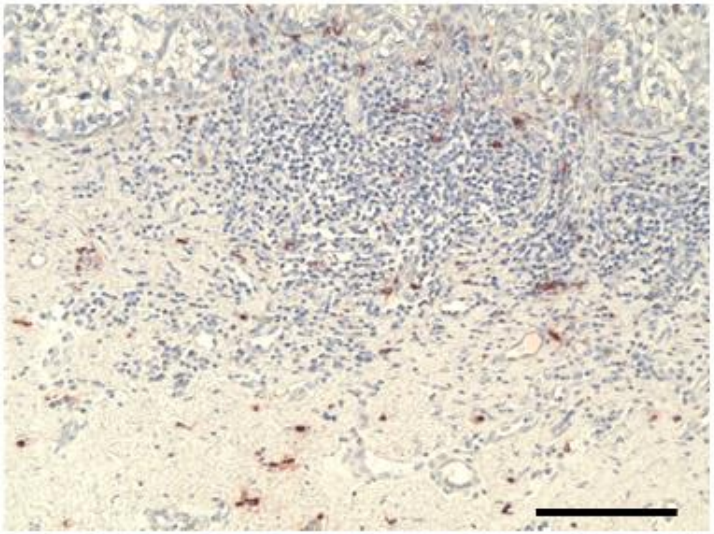

IHC staining for IL17
IHC staining
Quantitative analysis
a
b
c
d
non-invasive EMPD
invasive EMPD
e
IL-17 positive cells (%)
14
12
10
8
6
4
2
0
*
Non-invasive
Invasive

12.5. Fig. 5. IL-17 expression in invasive EMPD and non-invasive EMPD

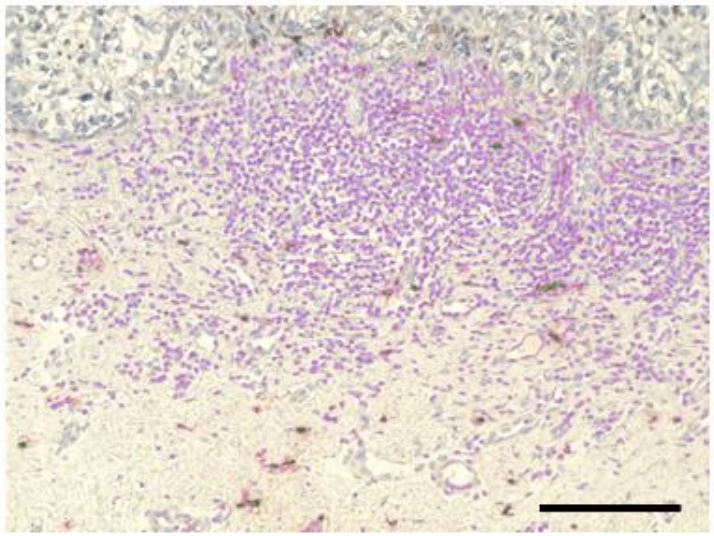
Sections of invasive EMPD and non-invasive EMPD lesions were deparaffinized and stained using anti-IL-17, the numbers of IL-17 expression cells expression cells were automatically counted by BZ-X800. The ratio of IL-17 positive cells to all tumor-infiltrating lymphocytes was significantly increased, from 6.4% to 9.3% ($P < 0.05$). Asterisks: * $p<0.05$ by Wilcoxon signed-rank test; Scale bars, 200 μm.

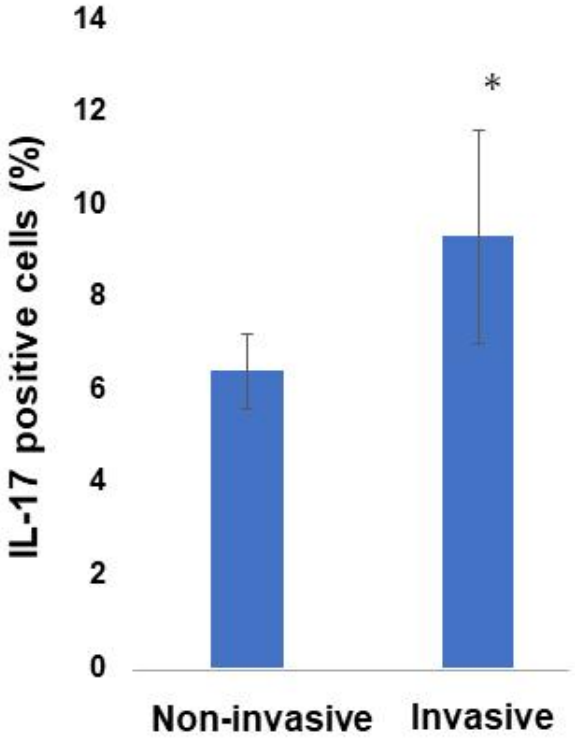
IHC staining for IL17

IHC staining



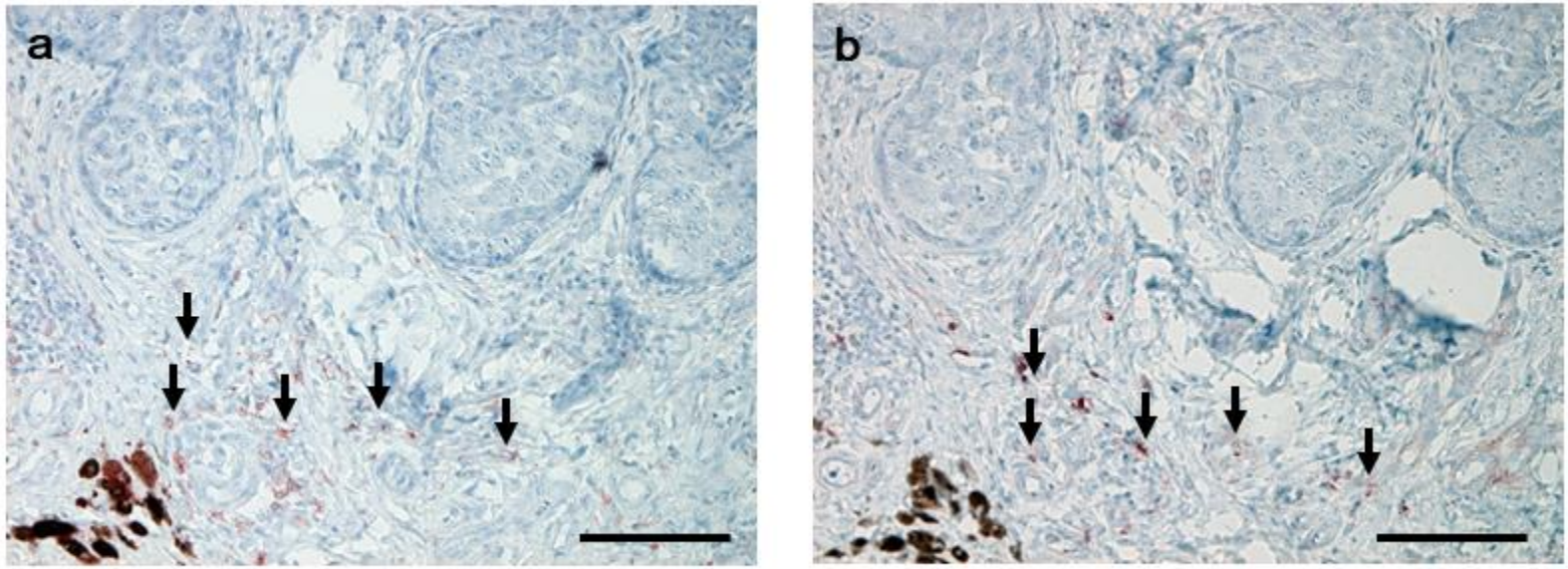
Quantitative analysis





12.6. Fig.6 The main population of LL-37 expressing cells CD163+TAMs.

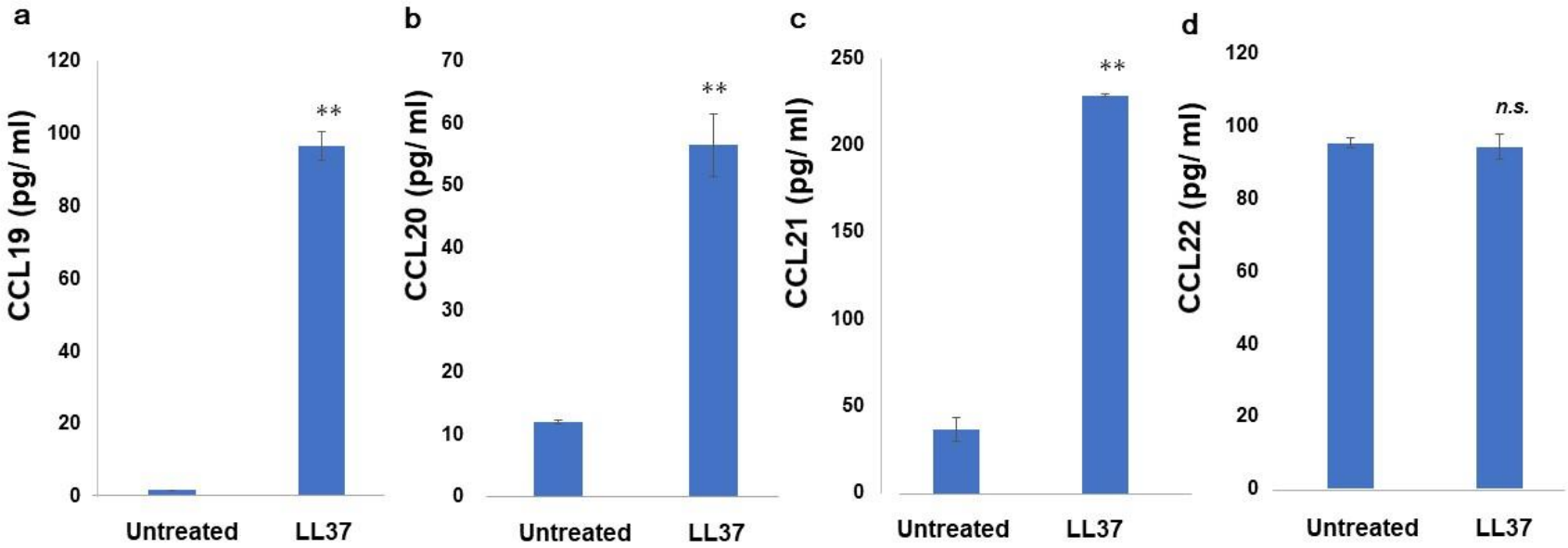
The serial sections of EMPD were deparaffinized and stained using anti-CD163 (a) and anti-LL-37 (b). IHC staining analysis of the serial sections in EMPD shows that CD163 positive expressing cells and LL-37 expressing cells were in the same location, and the main population of LL-37 expressing cells was CD163+TAMs. Scale bars: 100 μm (a, b).



CD163 LL37

12.7. Fig.7 LL-37 increased the production of CCL-19, CCL-20 and CCL-21 from monocyte-derived M2 macrophages.

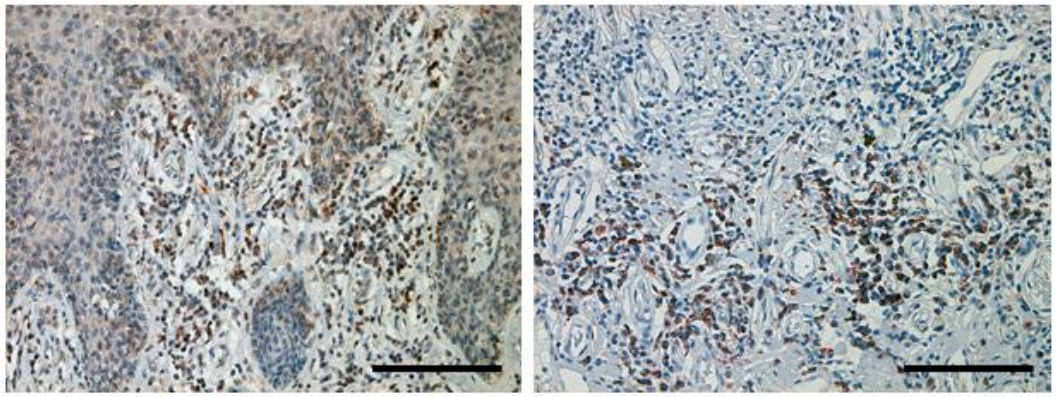
Culture supernatant from M2 macrophages was harvested as described in materials and methods, and measured by ELISA. Data from each donor were obtained from triplicate assays, and mean ± SD values were calculated. Representative data from at least three independent experiments are shown. *p <0.05, Mann-Whitney U-test; n = 5; *n.s.*, not significant.


a
CCL19 (pg/ ml)
120
100
80
60
40
20
0
**
Untreated
LL37
b
CCL20 (pg/ ml)
70
60
50
40
30
20
10
0
**
Untreated
LL37
c
CCL21 (pg/ ml)
250
200
150
100
50
0
**
Untreated
LL37
d
CCL22 (pg/ ml)
120
100
80
60
40
20
0
n.s.
Untreated
LL37

12.8. Fig.8 CD163+TAMs produce CCL-19 and CCL-21 in the lesional skin of EMPD

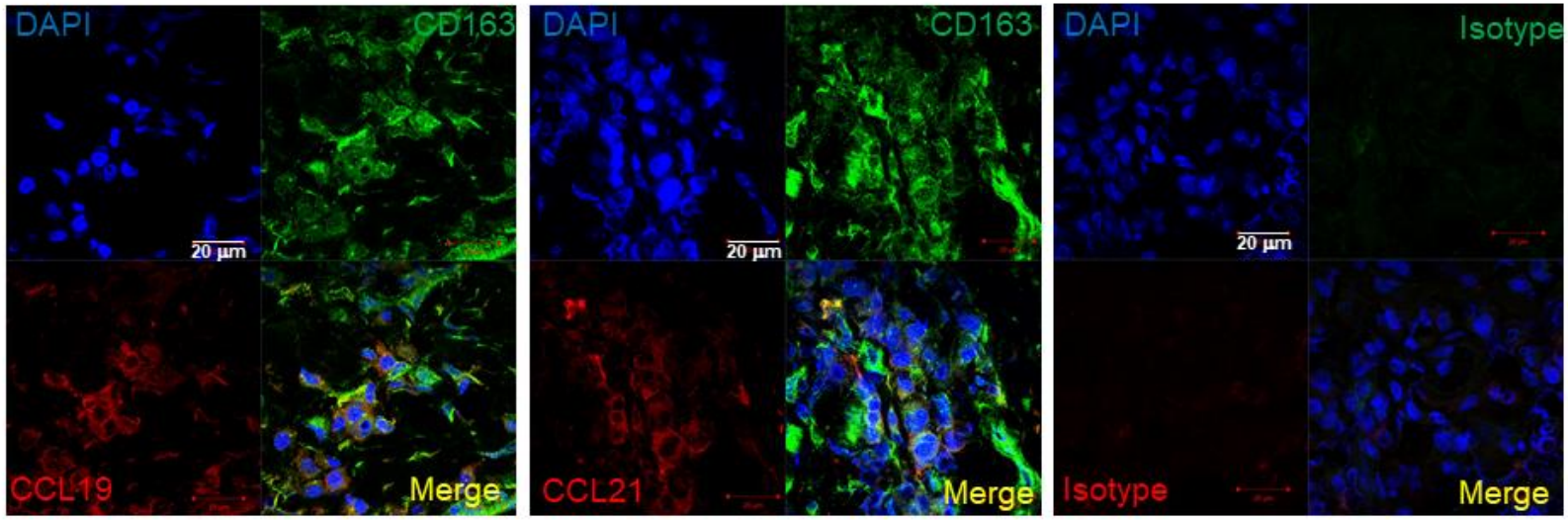
Representative Immunohistochemistry staining results of anti-CCL-19 and anti-CCL-21 expression in EMPD. Paraffin-embedded tissue samples from patients with EMPD were deparaffinized and stained using a combination of the anti-CCL-19 Ab or an anti-CCL-21 Ab, and developed with liquid permanent red (a). Immunofluorescence staining of invasive EMPD for CD163 (green), CCL-19 or CCL-21 (red), and DAPI (blue, nuclei). A merged image is also shown, with green and red combining into yellow (b). Scale bars: 100 μm (a), 50 μm (b).

a



CCL19 CCL21

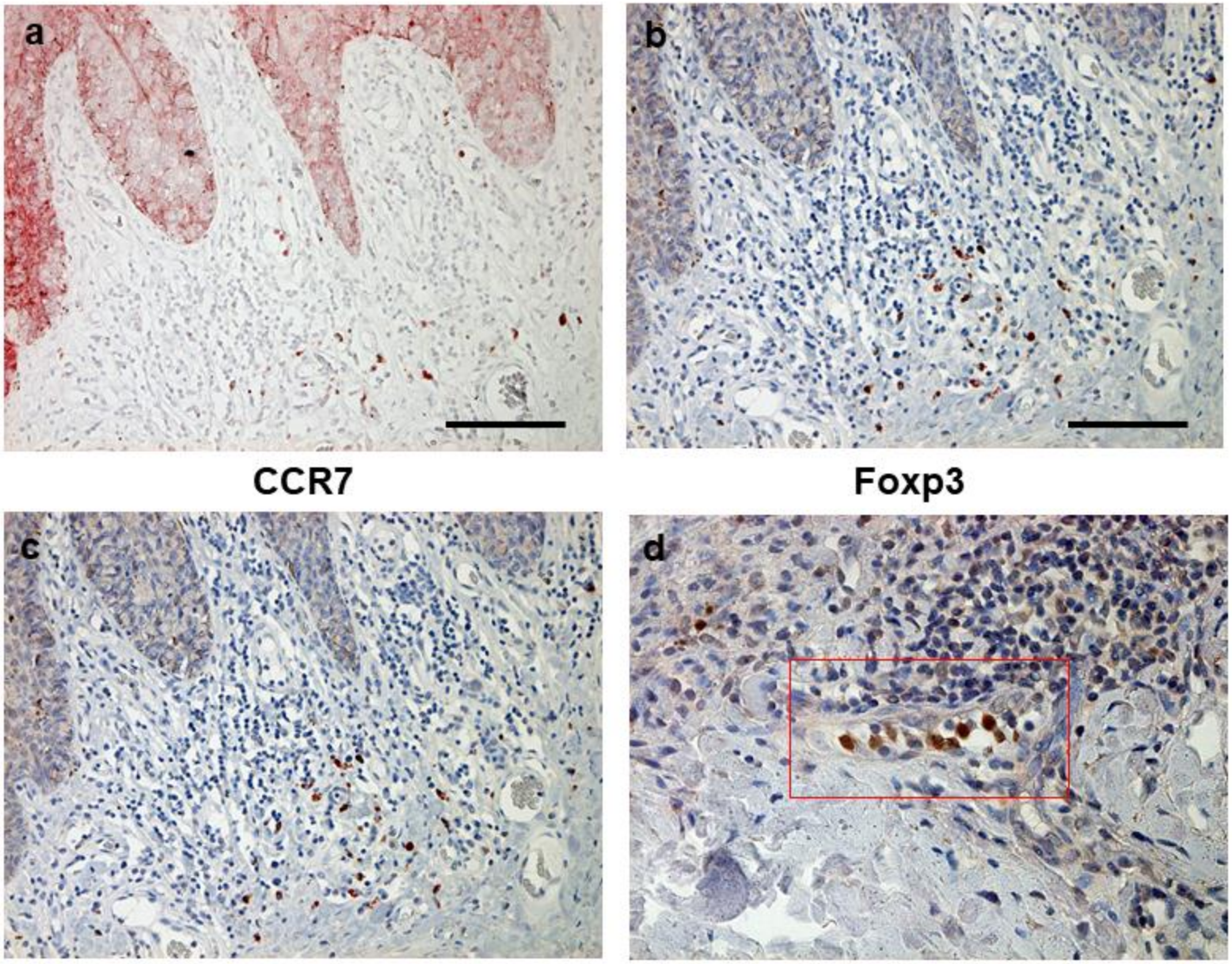
b



CD163/ CCL19 CD163/ CCL21 Isotype/ Isotype

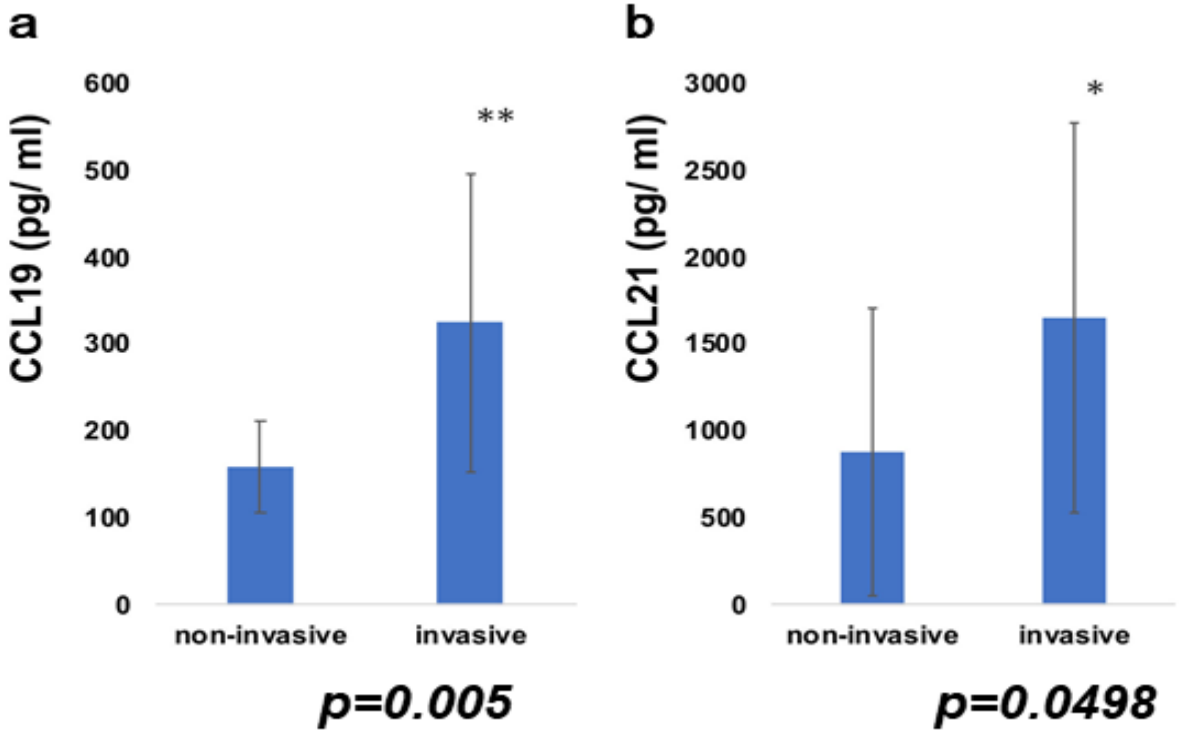
12.9. Fig.9 Immunohistochemical analysis of CCR-7 and Foxp3 expressions in lesional skin of EMPD

Sections of invasive EMPD lesions were deparaffinized and stained using anti-CCR-7 or anti-Foxp3 (a). Paraffin-embedded tissue samples from patients with invasive EMPD were deparaffinized and stained using a combination of the anti-Foxp3 Ab or an anti-CCR-7 Ab, and developed with liquid permanent red (a). Double IHC staining of Foxp3 and CCR-7 in the lesional skin of EMPD (b). Sections were developed with 3-Amino-9-ethylcarbazole for Foxp3 (red) and with 3, 3'-diaminobenzidine tetrahydrochloride for CCR-7 (brown). Scale bars, 100 μm (a-c), 50 μm (d).


a
b
CCR7
Foxp3
c
d
CCR7/ Foxp3
CCR7/ Foxp3

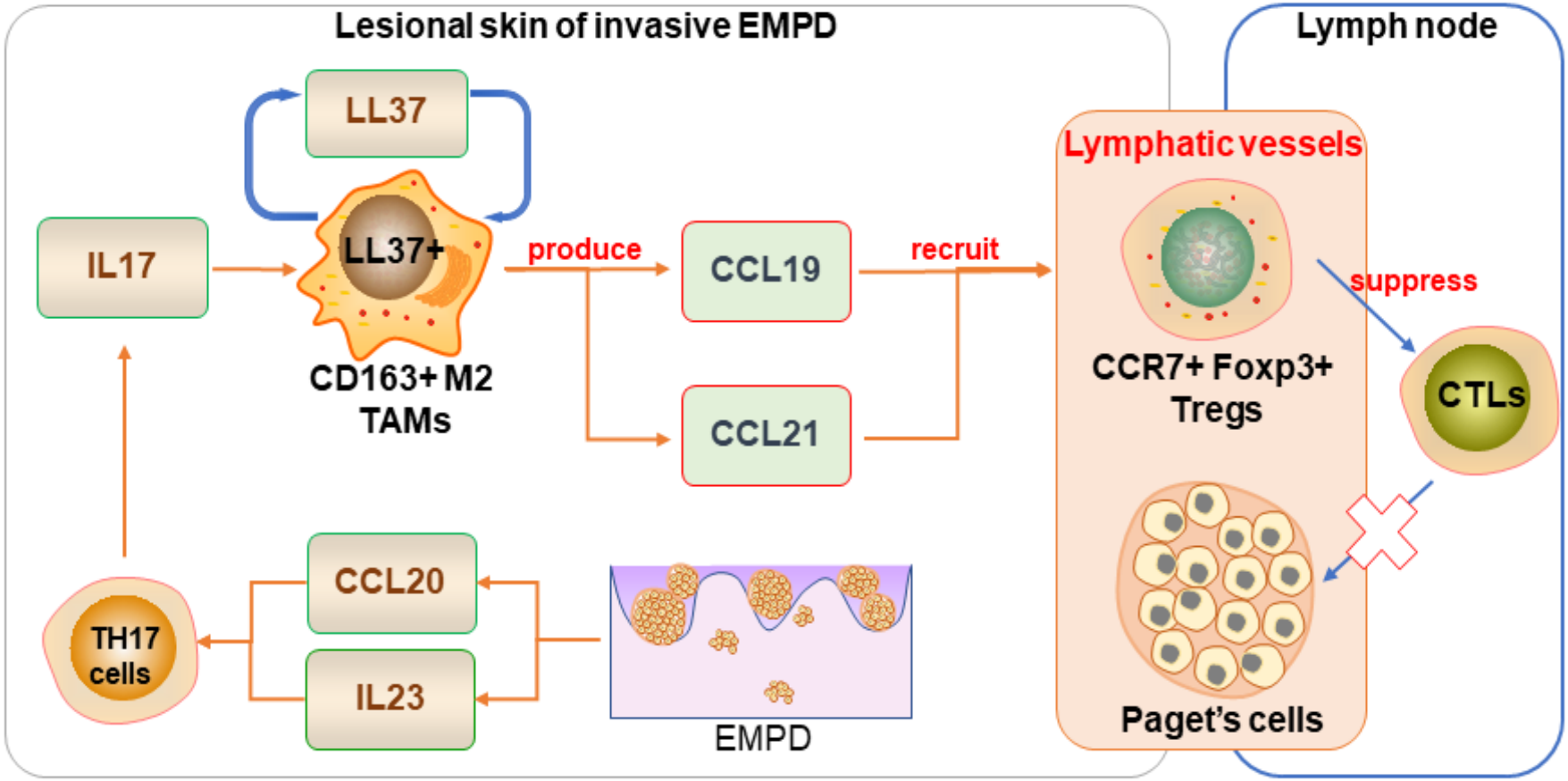
12.10. Fig.10 Serum levels of CCL-19 and CCL-21 in EMPD patients

Serum levels of CCL-19 (a) and CCL-21 (b) from 11 non-invasive EMPD patients and 15 invasive EMPD patients were examined by ELISA in Asterisks: ** $p<0.01$, * $p<0.05$ by Wilcoxon signed-rank test; *n.s.*: not significant.


a
CCL19 (pg/ ml)
600
500
400
300
200
100
0
**
non-invasive
invasive
p=0.005
b
CCL21 (pg/ ml)
3000
2500
2000
1500
1000
500
0
*
non-invasive
invasive
p=0.0498

12.11. Fig.11 Summary scheme

CCL-20 and IL-23 induces and recruits Th-17 cells to overproduce IL-17 in the microenvironment of lesional skin of EMPD. Since LL-37 is controlled by IL-17, LL-37 producing CD163+M2 TAMs accumulated in the lesional skin of invasive EMPD, thereby producing CCL-19 and CCL-21 to recruit CCR-7+Foxp3+Treg from the lymphatic vessels. The accumulation of CCR-7+Foxp3+Treg in invasive EMPD inhibits the immune cells transported from the lymphatic vessels, thereby promoting the lymphatic metastasis ability of Paget cells.


Lesional skin of invasive EMPD
Lymph node
LL37
IL17
LL37+
CD163+ M2
TAMs
produce
CCL19
CCL21
recruit
Lymphatic vessels
CCR7+ Foxp3+
Tregs
suppress
CTLs
Paget's cells
CCL20
IL23
TH17
cells
EMPD

13. Table

13.1. Table.1. Clinical information on EMPD patients

	NO.	Age	Sex	TNM Staging	Location
Non-invasive EMPD	Case 1	61-70	F	TisNoM0 stage 0	External genitalia, Mons pubis
	Case 2	51-60	F	TisNoM0 stage 0	External genitalia
	Case 3	71-80	F	TisNoM0 stage 0	External genitalia
	Case 4	81-90	F	TisNoM0 stage 0	External genitalia
	Case 5	61-70	M	TisNoM0 stage 0	Scrotum, penis
	Case 6	61-70	M	TisNoM0 stage 0	Scrotum, penis
	Case 7	81-90	M	TisNoM0 stage 0	Scrotum, penis
	Case 8	61-70	M	TisNoM0 stage 0	Peri anal
	Case 9	71-80	M	TisNoM0 stage 0	Scrotum, penis, inguinal
	Case 10	71-80	M	TisNoM0 stage 0	External genitalia
	Case 11	51-60	M	TisNoM0 stage 0	Scrotum
Invasive-EMPD	Case 12	71-80	F	T1N0M0 stage I	External genitalia
	Case 13	71-80	M	T1N0M0 stage I	Mons pubis
	Case 14	81-90	F	T1N0M0 stage I	External genitalia
	Case 15	81-90	M	T1N0M0 stage I	Penis
	Case 16	41-50	F	T1N0M0 stage I	External genitalia
	Case 17	81-90	F	T1N0M0 stage I	External genitalia

	Case 18	**61-70**	**M**	**T1N0M0 stage I**	**Scrotum, penis**
Invasive-EMPD	**Case 19**	**51-60**	**F**	**T1N0M0 stage I**	**External genitalia**
	Case 20	**71-80**	**M**	**T2N0M0 Stage II**	**Scrotum**
	Case 21	**61-70**	**M**	**T2N0M0 Stage II**	**Scrotum, penis**
	Case 22	**81-90**	**M**	**T2N0M0 Stage II**	**Penis**
	Case 23	**61-70**	**M**	**T2N0M0 Stage II**	**Scrotum**
	Case 24	**71-80**	**F**	**T2N2M0 Stage IIIB**	**External genitalia**
	Case 25	**61-70**	**M**	**T2N2M0 Stage IIIB**	**Scrotum, penis**
	Case 26	**51-60**	**F**	**T2N0M0 Stage II**	**Mons pubis**

13.2. Table 2: Antibodies used for immunohistochemistry

REAGENT or RESOURCE	RESOURCE	IDENTIFIER
Antibodies		
IHCPlus™ Monoclonal Mouse anti-Human CCL-19 / MIP3-Beta Antibody (IHC, WB)	LSBio	LS-B8194
Polyclonal Rabbit anti-Human CCL-21 / SLC Antibody (aa23-52, IHC, WB)	LSBio	LS-C168451
Anti-CCR-7 Antibody	LSBio	LS-C210538
LL-37 Antibody (D-5)	SANTA CRUZ	sc-166770
FOXP3 Antibody (2A11G9)	SANTA CRUZ	sc-53876
Goat Anti-Mouse IgG H&L (Alkaline Phosphatase)	Abcam	ab6790
Human CCL-22/MDC Antibody	R&D systems	MAB336
Liquid Mouse Monoclonal Antibody CD163	Novocastra™	NCL-L-CD163

13.3. Table 3: Solid Phase Sandwich ELISA Types

REAGENT or RESOURCE	RESOURCE	IDENTIFIER
Human CCL-19/MIP-3 beta DuoSet ELISA	R&D Systems	DY361
Human CCL-20 /MIP-3 alpha DuoSet ELISA	R&D Systems	DY360
Human CCL-21/6Ckine DuoSet ELISA	R&D Systems	DY366
Human CCL-22/MDC DuoSet ELISA	R&D Systems	DY336