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独立行政法人日本学術振興会 理事長 殿

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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地： リューベック （国名： ドイツ ）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
自己免疫性水泡症における画期的マウスモデルの樹立と病態解明

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部局名： 皮膚科学研究室

5. 所期の目的の遂行状況及び成果…書式任意 **書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

Introduction

My project focuses on the elucidation of the pathogenesis of pemphigoid diseases (PD), especially by analyzing the binding of autoantibodies to the keratinocyte, the pemphigoid-targeted cell. This project is the initial step for the establishment of a disease-specific mouse model in PD.

PD are a heterogeneous group of autoimmune subepidermal blistering diseases, which are clinically characterized by cutaneous or mucosal blisters. PD are caused by autoantibodies targeting the dermal-epidermal junction (DEJ) proteins such as BP230, BP180, Laminin 332 and COL7.

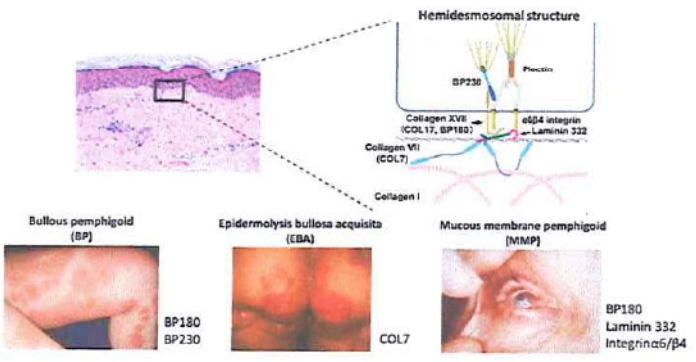

Figure 1. The structure of components of the dermal-epidermal junction (DEJ) and clinical manifestations of pemphigoid disease (PD). Bullous pemphigoid (BP) patients have autoantibodies against BP180 and/or BP230. Epidermolysis bullosa acquisita (EBA) is caused by autoantibodies against COL7. Mucus membrane pemphigoid (MMP) autoantibodies target BP180, laminin 332, or integrin α6β4. Image from *J Dermatol Sci. 73:179*

Bullous pemphigoid (BP) is the most common PD caused by autoantibodies directed against two hemidesmosomal proteins, type XVII collagen (BP180; COL17) and BP230. In BP, the non-collagenous (NC16A) domain is known to be the major pathogenic epitope on BP180 (**Figure 1**). Patients with BP typically show widespread skin blister formation accompanied with erythemas and severe itch. Other PD include mucous membrane pemphigoid (MMP) and epidermolysis bullosa acquisita (EBA, **Figure 1**). In MMP, autoantibodies target different molecules including COL17, Laminin 332, type VII collagen (COL7) and sometimes integrin α6/β4. MMP patients predominantly present with mucosal lesions. EBA is characterized and caused by autoantibodies against COL7 which is a major component of anchoring fibrils. Clinically, EBA is characterized by tense blisters and scaring on the skin and the mucosa.

It is still not fully elucidated, how autoantibody binding to different PD autoantigens leads to such diverse pathologies. Even though BP180 is a common antigen in both BP and MMP, these two diseases target distinct epitopes on BP180 (BP targets NC16A domain and MMP targets the C-terminus), and manifest different clinical findings or treatment outcomes (Figure 1). In addition, no animal model for anti-BP180, anti-α6 integrin, and β4 integrin MMP is available, even though experimental models for BP and EBA have been established in several laboratories. In order to elucidate the possible reasons why a MMP mouse model targeting an antigen other than Laminin 332 is difficult, we focused on the specific underlying pathogenesis in different pemphigoid diseases. We hypothesized that in their target cells, i.e. keratinocytes, differential kinetics events are triggered by binding of different PD autoantibodies, which lead to diverse clinical manifestations.

Hence, by understanding the immunological event triggered by PD autoantibody binding, we will expand the knowledge of PD pathogenesis, which will be the basis to develop novel mouse models for these diseases. To reach this goal, we focused on mediator releases and kinome activities triggered by PD autoantibody binding to the keratinocytes, which have not been focused on in previous studies in PD.

Result

1)Elucidation of the PD autoantibody binding-induced kinome in keratinocytes

The host lab has recently obtained the PamGene kinase microarray system, allowing to measure the activity of 176 kinases in tissue or cell lysates. More specifically, this system requires very small quantities of lysates (1-5 μg protein per chip) to measure the activity of kinases in a wide range of cell lines or tissues. This is accomplished by incubating the sample lysates across 144 tyrosine (PTK) or 196 serine / threonine (STK) kinase peptide substrates immobilized on the surface of the PamGene microarray. Kinases present in the lysates will phosphorylate the peptide substrates which are subsequently detected using fluorescently labelled antibodies (**Figure 2a**). This, among others, gives us the unique opportunity for pathway elucidation in lysates obtained from the keratinocytes incubated with PD autoantibodies. The applicant significantly contributed to the establishment of this system in the lab. To better understand kinase regulation triggered by PD autoantibodies, we incubated the immortalized keratinocyte cell line HaCaT with affinity-purified PD patient IgG (anti-BP180-NC16A from BP patients or anti-COL7 IgG from EBA patients) using either serum or immunoapheresis material. HaCaT incubated with normal human IgG, with no detectable PD autoantibodies, served as negative controls. To ensure that the presence of endotoxins such as LPS has an impact on kinase activity, absence of endotoxins in all media, reagents and IgG preparations was ensured. We analyzed the PTK and STK kinase activities, detecting a total of 108 activated kinases following incubation of keratinocytes with either BP IgG or EBA IgG. Among those, 61 kinases were selectively activated when incubated with BP IgG, while 81 kinases were activated exclusively by incubation

with EBA IgG (**Figure 2b**).

We made the rather unexpected observation that the kinase activity between BP IgG or EBA IgG incubated HaCaT cells, is strikingly different. Among these differences is the time kinetics of kinase activation, whereby EBA IgG incubated HaCaT cells seemed to have a later peak of kinase activation than those incubated with BP IgG (**Figure 2b-c**). Interestingly, kinases that are significantly up or down regulated were also different. These findings suggest that a different kinase network may contribute to the disease specific pathogenesis in BP and EBA. To further depict the protein composition, the kinase activity measured after a 5 min incubation with either BP IgG or EBA IgG was analyzed using Proteomaps (https://www.proteomaps.net), which show the quantitative composition of proteomes with a focus on protein function. They are built automatically from proteome data and are based on the KEGG pathways gene classification (https://www.kegg.jp). Each protein is shown by a polygon and functionally related proteins are arranged in common regions. The Proteomap of BP IgG shows an abundant expression of the Erb B signaling pathway which regulates diverse biologic responses, including proliferation, differentiation, cell motility, and survival. By contrast, the stimulation of EBA IgG induces the expression of abundant MAPK signaling, which is among the most compelling therapeutic targets for oral drug development for cancer and chronic inflammatory diseases, due to the key role of cell signaling in cancer and immune cells (**Figure 2c**). These results further underscore that incubation with either PD IgG induces a significant alteration of kinase activity in keratinocytes, which is, however, quite distinct when contrasting the kinase activity of BP IgG to EBA IgG incubated HaCaT cells.

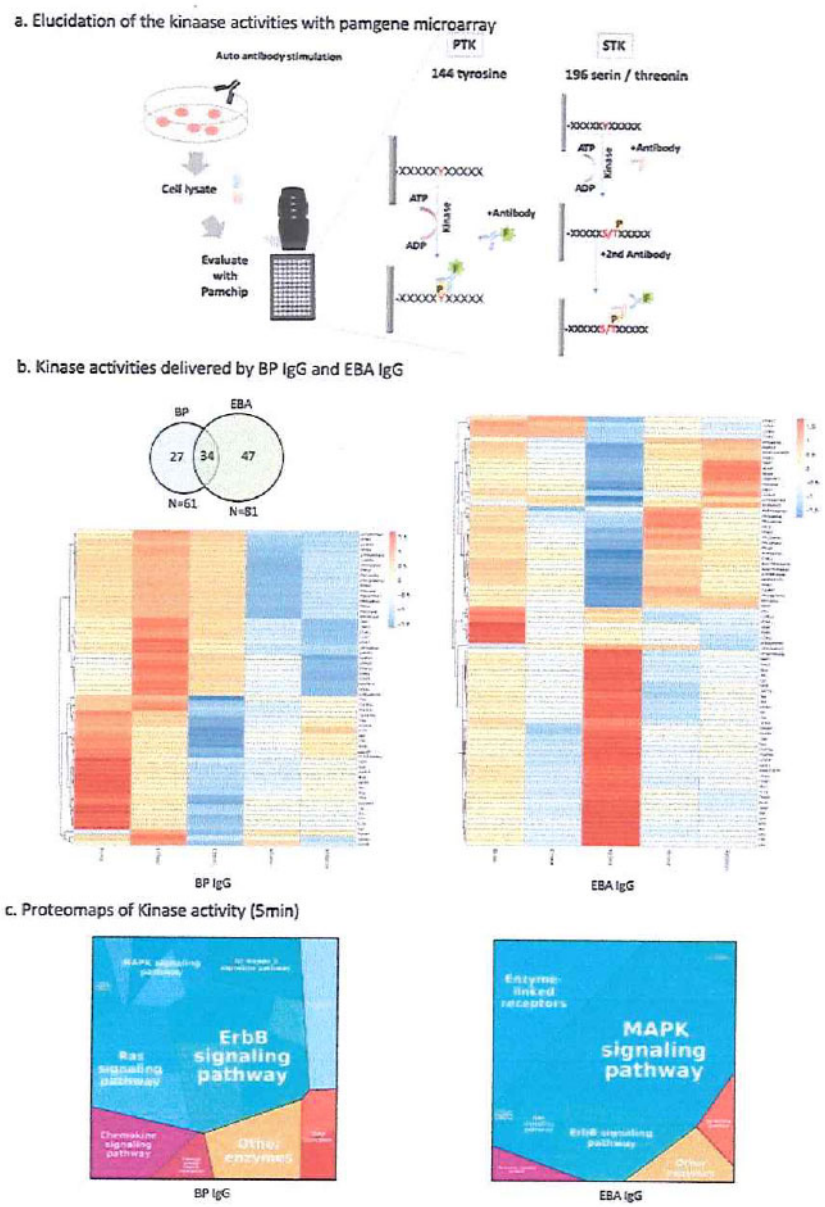

Figure 2. BP IgG and EBA IgG induce distinct kinase activities in keratinocytes. **a.** PD autoantibodies were purified from immune apheresis (IA) material of BP or EBA patients (n=3 for each disease) using NHS-Sepharose columns and coupling proteins (BP180-NC16A for BP, COL7 for EBA). Specific autoantibodies were eluted using 0.1 M glycin buffer. Keratinocytes were stimulated with 40µg/ml IgG autoantibodies (BP IgG, EBA IgG) for 5min, 15min, 30min, 60min, and 360min. The cell lysates were collected in M-PER reagent containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo, Waltham, MA). The protein concentrations were measured using the BCA kit (Thermo) and were applied at equal amounts of protein to the specific Pamchip for performing Protein Tyrosine Kinase (PTK) assay and Serine / Threonin Kinase (STK) assay according to the company's protocol. Images were captured and quantitation performed using the BioNavigator v. 5.1 (PamGene). **b.** Venn diagram shows common or unique kinases activated by BP and EBA IgG. Heatmaps show kinases detected by PamGene. Analyses were compered between BP and EBA at/for each timepoint of stimulation. **c.** Proteomaps depict the detected kinases in the 5min stimulation with BP IgG and EBA IgG. The proteomap of BP IgG shows the abundant expression of Erb B signaling pathway. By contrast, the stimulation of EBA IgG induces the expression of abundant MAPK signaling pathway.

2) Elucidation of the PD autoantibody binding-induced secretome in keratinocytes

Previous work showed a time and dose-dependent release of IL-6 and IL-8 from BP IgG incubated keratinocytes (Schmidt E, et al. J Invest Dermatol 2000).

We recently showed that these 2 cytokines, as well as the complement component 5 (C5), are also released by keratinocytes upon incubation with EBA IgG (**Figure 3**).

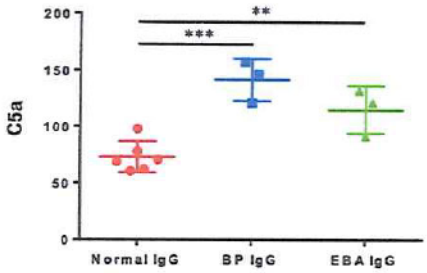

Figure 3. C5a secretion from HaCaT cell incubated with PD IgG. HaCaT cells were incubated with BP IgG, EBA IgG or normal human IgG for 12 hours, then supernatants were collected. The amounts of C5a in supernatants were measured by ELISA. (One-way ANOVA. **: $p<0.005$, ***: $p<0.0005$).

To obtain an unbiased and more comprehensive overview of the proteins that are secreted by keratinocytes after incubation with either BP IgG or EBA IgG, we used the Olink platform (Uppsala, Sweden) to determine the expression of close to 300 different proteins in the supernatants of HaCaT cells incubated with either BP IgG, EBA IgG, or normal human IgG (Olink panels: Inflammation, Cell Regulation, Metabolism, Immune response and Organ Damage).

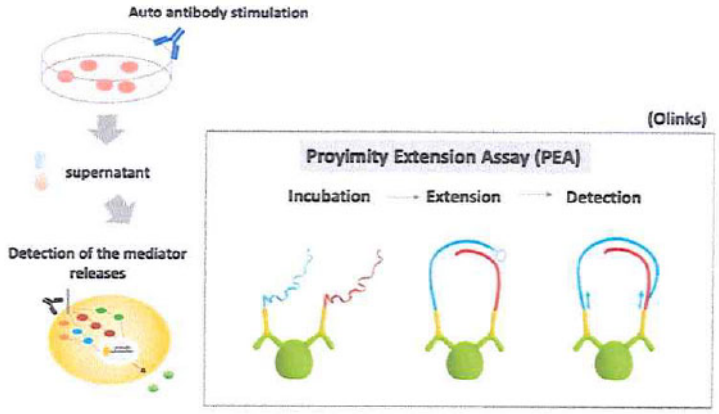

Figure 4. Proximity Extension Assay (PEA) technology.

Cells incubated with normal human IgG, with no detectable PD autoantibodies, were sent to a commercial supplier of proteomics analysis, Olink (Uppsala, Sweden), which uses the Proximity Extension Assay (PEA) technology.

Hereby a pair of oligonucleotide-labeled antibodies for the same target antigen (but with different epitopes) are bound pair-wise to the target protein present in the supernatant. Next, but only when the two probes are in close proximity, a new PCR target sequence is formed by a proximity-dependent DNA polymerization event. The thereby resulting sequence is subsequently detected and quantified using standard real-time PCR.

Using the Olink technology, we identified 34 proteins to be secreted by keratinocytes incubated with either BP IgG or EBA IgG. Furthermore, 11 and 56 proteins were uniquely secreted by keratinocytes incubated with BP IgG or EBA IgG, respectively (**Table 1**).

BP and EBA (n=34)	Only BP (n=11)	Only EBA (n=56)	
uPA	STX8	SUMF2	CAPG
CCDC80	NOMO1	MCP-1	DAPP1
TACSTD2	MAGED1	ACP6	BCR
OPG	VEGFA	TNFRSF10A	TINAGL1
MMP-1	KAZALD1	SERPINB6	TP53
KLK10	LIF	PPP1R2	LGALS7
IL-1 alpha	COL4A1	FOSB	IRAK1
STC1	SDC4	PRDX1	CD164
ALDH3A1	LYAR	QDPR	MAP2K6
EIF4G1	PGF	4E-BP1	DCTN2
IGSF3	PAG1	SERPINB8	STX6
MMP-10		IL18	MAEA
TOP2B		CD40	RCOR1
MCFD2		HEXIM1	ADGRE2
NBN		TIGAR	ZBTB17
IL8		METAP1D	PRDX6
CXCL1		ADA	DNAJB1
SRPK2		DFFA	PRDX3
ITGB6		NECTIN2	MGMT
HDGF		FAM3C	AREG
TGF-alpha		CSF-1	CANT1
CA13		STAMBP	TYMP
CASP-8		CDCP1	IL6
THOP1		MVK	YES1
DDX58		BAG6	DCTN1
NADK		SNAP23	KYAT1
CXCL5		AHCY	USP8
CRKL		COMT	PRDX5
AMIGO2			
APEX1			
ENAH			
FKBP4			
IL-18R1			
NPDC1			

Table 1 List of proteins secreted from HaCaT cells incubated with BP IgG or EBA IgG. Olink technology identified 34 proteins to be secreted in common by keratinocytes incubated with either BP IgG or EBA IgG. Furthermore, 11 proteins were uniquely secreted with the incubation of BP IgG and 56 proteins were uniquely secreted with the incubation with EBA IgG, respectively. The order of the proteins corresponds to the observed fold-change; i.e. the first protein in each column showed the greatest fold-change.

In addition to the Olink technology, we also used "classical" ELISA to determine if incubation with anti-BP180-NC16A, keratinocytes can release C5 because keratinocytes have been noted to be able to produce C5[23], and the C5a-C5aR-axis significantly contributes to PD pathogenesis[24,25]. Indeed, incubation of HaCaT cells with either BP IgG or EBA IgG led to an increased expression of C5a in the supernatant (**Figure 3**). This indicates that proteins released by the keratinocytes after incubating with PD autoantibodies must be able to cleave C5 into C5a and C5b.

Taken together, our project provides detailed insights into the effects of PD autoantibodies on keratinocytes. The identified kinome and secretome is, however, based on techniques using sample size numbers that require validation. Next, we will identify the mechanisms how PD autoantibodies induce the alterations in the keratinocyte kinome and secretome. Ultimately, causal relationships between the kinome and secretome will be established and the functional impact of the secretome will be characterized.