

TITLE PAGE

Letter to the Editor

Local inflammation exacerbates cutaneous manifestations in a murine autoimmune pemphigus model

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This work was supported by the Japan Society for the Promotion of Science KAKENHI (263395), Grants-in-Aid for Scientific Research 15H05790, 15H1155, 15K15417, Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology (PRESTO) (16021031300), and Japan Agency for Medical Research and Development (AMED) (16ek0410011h0003, 16he0902003h0002).

Conflicts of interest: none.

Word count: 999.

Capsule summary: Local inflammation enhances autoantibody deposition in the skin and exacerbates murine pemphigus manifestations as a representative of autoantibody-mediated disorders, therefore prevention of excessive external stimulants might be important for the disease management.

Key words

autoantibody-mediated disorders, autoimmune blistering disorders, pemphigus, desmoglein; antibody, inflammation, histamine

Abbreviations

Dsg: Desmoglein

DIF: Direct immunofluorescence

FITC: Fluorescein isothiocyanate

PMA: Phorbol myristate acetate

IgG1-MFI: The mean fluorescent intensity of immunoglobulin G1

UVB: ultraviolet B

To the Editor:

Autoantibodies directed against antigens in extravascular tissues must pass through the blood vessel wall to exert their pathogenicity. How their extravasation is regulated *in vivo* remains largely unknown. Pemphigus is one of such autoantibody-mediated disorders, which is caused by pathogenic autoantibodies against epidermal desmosomal cadherins, desmoglein (Dsg) 1 and Dsg3. Because these autoantibodies can directly disrupt epidermal cell-cell adhesion¹, their concentration in the epidermis would be linked to the disease severity. Such anti-Dsg antibody (Ab) deposition in the epidermis has been evaluated by direct immunofluorescence (DIF) assay in the skin biopsy specimens. Besides, the elicitation of blistering after external stimuli such as mechanical trauma or sun exposure has been reported in pemphigus patients^{2,3}. These observations imply that the local inflammation, which permits extravasation of macromolecules⁴, might also promote extravasation and subsequent deposition of autoantibodies in the skin. Herein, we tested this hypothesis by using a murine pemphigus model.

We first evaluated the dynamics of epidermal Ab deposition in inflamed and non-inflamed skin in mice after injecting fluorescein isothiocyanate (FITC)-conjugated AK18, a non-pathogenic anti-mouse Dsg3 Ab that belongs to the immunoglobulin (Ig) G1 subtype⁵. We used this non-pathogenic Ab to avoid keratinocyte dissociation and local inflammation that may hinder the subsequent analysis. Intravital observation by two-photon microscopy demonstrated that epidermal AK18 deposition occurred gradually over 6 h in the inflamed ears treated with topical phorbol myristate acetate (PMA), whereas no deposition was observed in the untreated ears (**Figure 1A**), suggesting that local inflammation enhances AK18 deposition.

For more precise evaluation, we employed a flow cytometric analysis. Twenty-four hours after intravenous injection of AK18 simultaneously with PMA application to the ear skin, the concentration of AK18 in the ear epidermis was quantified by measuring the mean fluorescent intensity (MFI) of IgG1 in CD45⁻ E-cadherin⁺ keratinocytes (hereafter referred to as IgG1-MFI) (**Figure 1B and E1A-B**). IgG1-MFI was significantly increased in the PMA-painted ears compared to the untreated ears (**Figure 1C**). Neither the expressions of Fcγ receptors nor IgG1-MFI following control-IgG1 injection was elevated on keratinocytes after PMA application, suggesting that this assay specifically detects AK18 deposition in the epidermis (**Figure E1C-D**). In both the untreated and PMA-painted ears, IgG1-MFI positively correlated with the dose of

AK18 injected (1 to 1000 μ g) (**Figure 1D**).

In the PMA-painted ears, 10 μ g of intravenous AK18 injection to mice seemed to be sufficient to induce the equivalent IgG1-MFI in the untreated ears of mice that received 100 μ g of AK18 (**Figure 1D**). Consistently, DIF assay revealed epidermal Ab deposition in the PMA-painted ears that received as little as 5 μ g of AK18, whereas Ab deposition was undetectable in the untreated ears of mice that received as much as 50 μ g of AK18 (**Figure 1E**). Similar enhancement in Ab deposition in the PMA-painted ears was observed irrespective of IgG subclasses (IgG1 or IgG4) when injecting serums from two pemphigus patients (**Figure 1F and E2**). At least 100 μ l of each serum was required to obtain apparently positive DIF results in the untreated ears, while positive DIF results were observed in the PMA-painted ears when injecting 20 μ l of each serum. Taken together, these findings allowed us to consider that local skin inflammation can enhance the autoantibody deposition in the epidermis *in vivo*.

To evaluate epidermal Ab deposition under more physiological skin inflammations, we employed other inflammatory stimuli, such as a single painting of hapten (**Figure 2A**) and tape-stripping (**Figure 2B**). These treatments were sufficient to increase IgG1-MFI. In addition, we irradiated mouse ears with ultraviolet B (UVB) and demonstrated a positive correlation of IgG1-MFI with the UVB irradiation dose ($r = 1.00$) (**Figure 2C**) and with the intensity of skin inflammation as evaluated by ear swelling ($r = 0.90$) (**Figure 2D**). These results indicate that deposition of autoantibody in tissue is correlated with the severity of local inflammation in mice.

Lastly, to confirm that local inflammation predisposes to the development of the pemphigus phenotype, we used a pathogenic anti-mouse Dsg3 Ab, AK23. As previously reported⁵, administration of a sufficient dose of AK23 resulted in hair loss in mice (>80-100 μ g, **Figure E3**). In contrast, hair loss and epidermal Ab deposition was limited to the PMA-painted area when mice received a sub-pathogenic dose (50 μ g) of AK23 (**Figure 2E and 2F**). These observations demonstrate that pemphigus lesions tend to occur at the local inflammatory site in mice.

We have previously demonstrated that the histamine-induced vascular hyperpermeability evokes the extravasation of macromolecules *in situ*⁴. In this context, we hypothesized that cutaneous histamine prick⁶ might be useful for enhancing the diagnostic sensitivity of DIF assay. We evaluated Ab deposition in the mouse ear after histamine prick with intravenous AK18 injection. As expected, IgG1-MFI was

increased by histamine prick, but not by vehicle-prick (**Figure 2G**). This result suggests that preceding histamine prick is useful for enhancing Ab deposition in the skin.

In summary, our results in mice suggest that local inflammation might also enhance anti-Dsg3 Ab deposition in the skin in pemphigus patients. Thus, taking skin biopsies from perilesional erythematous (presumably inflammable) areas in autoimmune blistering diseases might be beneficial to increase the DIF sensitivity. It might also explain the induction of blisters after external stimuli such as sun exposure, the pathogenesis of which remains unclear^{2,3}. Likewise, the predisposition of pemphigus skin lesions on the back as well as intertriginous and seborrheic areas⁷ might reflect the existence of multiple external stimulants, including frictions and mechanical pressure. This increase in autoantibody deposition (**Figure E4**) might be common for antibody-related autoimmune conditions and related to their symptoms, such as the sun exposure-induced skin eruptions in lupus erythematosus⁸. Furthermore, our preliminary study demonstrated that a histamine pricking, which is commonly used in clinic as a positive control for type I hypersensitivity^{6,9}, may improve the diagnostic sensitivity of DIF assay when administered prior to skin biopsy (data not shown). A cutaneous histamine prick might also assist in diagnosis of patients especially whose pemphigus lesions are confined to the oral mucosa, where a taking biopsy specimen is often hard to conduct.

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150 **Acknowledgement**

151 We thank Dr. Masayuki Amagai (Keio University) for kindly giving us AK18 and
152 AK23 hybridomas.

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Figure legends**Figure 1. Anti-Dsg3 Ab deposition in the untreated and PMA-painted skin**

A, Time-lapse images and Δ FITC-MFI of epidermis in the PMA-painted (PMA) or untreated (UT) ear after FITC-labeled AK18 injection. **B**, Identification of IgG1 among keratinocytes. **C**, IgG1-MFI after 20 μ g of AK18 or control IgG1 injection. **D**, Δ IgG1-MFI against AK18 injection doses. **E-F**, DIF after injection of AK18 (**E**), or serum from a healthy donor or a pemphigus patient (**F**).

Figure 2. Anti-Dsg3 Ab deposition under various skin inflammatory conditions

A-C, IgG1-MFI of hapten-painted (**A**), tape-stripped (**B**), and UVB-irradiated ears (**C**) compared to that of untreated ears. **D**, A plot of ear swellings against IgG1-MFI in **C**. **E-F**, Hair loss (**E**) and AK23 deposition (**F**) limited to the PMA-painted area. **G**, IgG1-MFI of the ear after a histamine- or vehicle (Veh)-prick. Precise information is located in METHODS of Online Repository.

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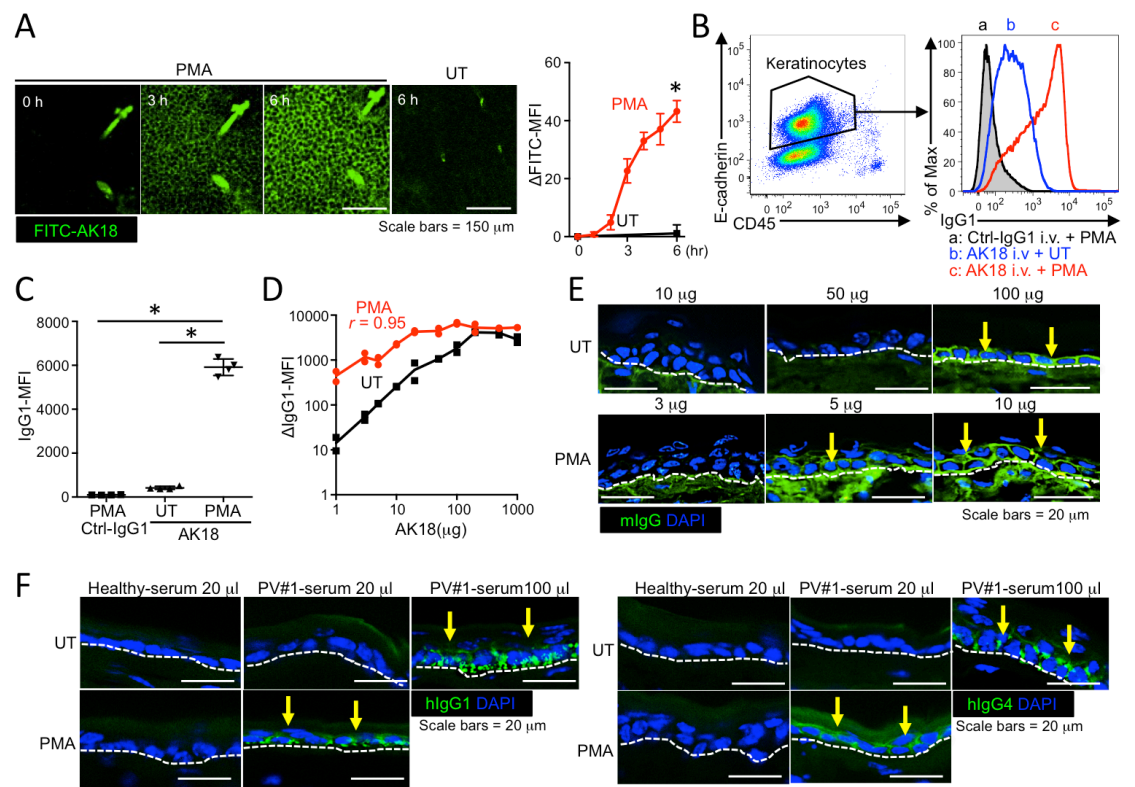


Figure 1

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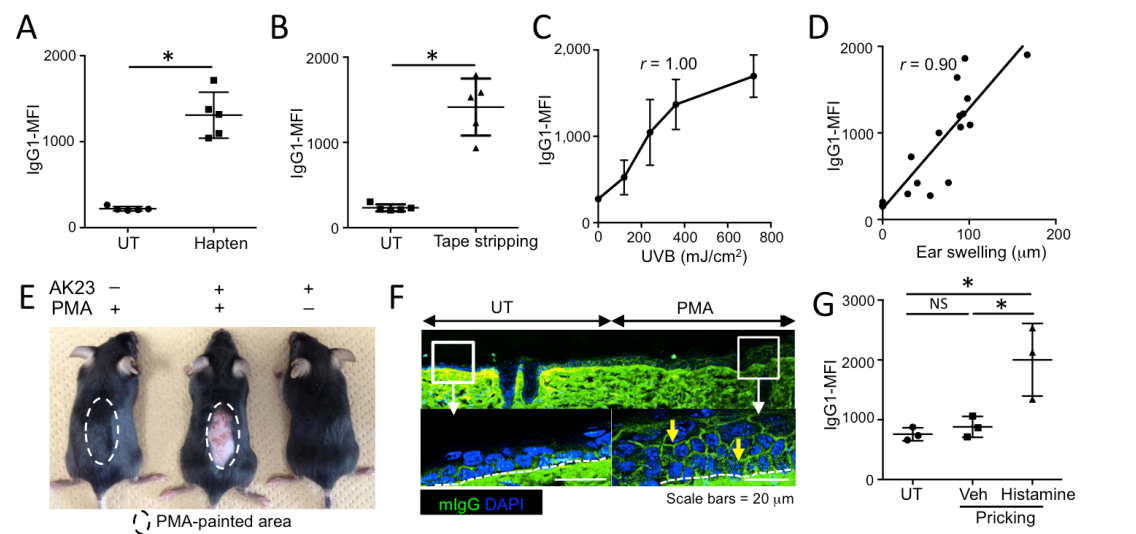


Figure 2

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METHODS

Preparation of epidermal cell suspension for a flow cytometric analysis and calculation of IgG1-MFI.

AK18 or control mouse IgG1 κ (Biolegend, SanDiego, CA) diluted in 500 μ l phosphate buffered saline (PBS) were injected via tail vein. In every examination otherwise indicated, the ear skins were collected 24 h after intravenous injection of AK18, and dorsal halves were floated for 30 min at 37°C on 5 mg/ml dispase II (Wako) dissolved in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum. The epidermis was then manually separated from the dermis and floated on 0.25% trypsin-EDTA for 8 min at 37°C and filtered through 70- μ m cell-strainer mesh (BD Bioscience, SanDiego, CA). The epidermal suspensions were stained with fluorochrome-conjugated antibodies to E-cadherin, CD45, and mouse IgG1 (BD Bioscience). Cells were analyzed with FACS LSR Fortessa flow cytometric system (BD Bioscience) and FlowJo software (Tree Star, Ashland, OR), and the mean fluorescence intensity of IgG1 (IgG1-MFI) was calculated in E-cadherin⁺ CD45⁻ epidermal keratinocytes. Δ IgG1-MFI indicates the changes in MFI from vehicle control.

Time-lapse image acquisition by two-photon microscope.

5 mg/ml of fluorescein isothiocyanate (FITC) in dimethyl sulfoxide, 1 M sodium bicarbonate buffer at pH 9.1, and 1-2 mg/ml of AK18 were reacted for two hours at RT. FITC-conjugated AK18 were obtained after several dialyses. Phorbol myristate acetate (PMA) was applied to the mouse ear to evoke inflammation 24 h before intravenous injection of 100 μ g of FITC-labeled AK18. Mice were positioned on the heating-plate on the stage of a two-photon microscope, IX-81 (Olympus, Tokyo, Japan) and their ear lobes fixed beneath cover slips with a single drop of immersion oil. The tail vein was cannulated using polyethylene tubing (PE-10: Becton, Dickenson and Co, Franklin Lakes, NJ) with a 30-gauge disposable needle (Dentronics, Tokyo, Japan) connected to a syringe filled with FITC-conjugated AK18. The time-course of *in-vivo* AK18 deposition to the epidermal keratinocytes was evaluated after injection. To calculate Δ FITC-MFI, the changes in MFI from 0 h were evaluated in three epidermal areas.

Direct immunofluorescence (DIF) analysis.

As for DIF staining, the ears of mice were collected 24 h after intravenous injection

(otherwise indicated) of AK18, or serums from PV patients or a healthy donor, fixed in 4% paraformaldehyde in PBS, and embedded in O.C.T. compound (Sakura Finetek, Torrance, CA) for frozen sectioning. 5- μ m slices were prepared and were incubated for 30 min at room temperature with goat anti-mouse IgG Alexa fluora 546 (Invitrogen), after treatment with Image-iT FX Enhancer (Invitrogen). Slices were mounted with anti-fade mounting reagent with DAPI (Invitrogen). In each picture, yellow arrows represent intercellular autoantibody deposition. Blue represents nuclei stained by DAPI. White dotted lines represent the border between the epidermis and the dermis.

DIF analysis using serums from pemphigus patients.

Serums from two pemphigus vulgaris patients (PV#1 and PV#2), who were positive for anti-Dsg3 Ab but not anti-Dsg1 Ab, were collected for the analysis. Among the IgG1-IgG4 subclasses, PV#1 was positive for the IgG1 and IgG4 subclasses. PV#2 was positive for the IgG4 subclass. With intravenous injection of each serum of PV#1 and PV#2 to adult mice via tail vein, we determined that at least 100 μ l of each patient's serum was required to obtain apparently positive DIF results in the untreated ears. In contrast, positive DIF results were observed in the PMA-painted ears of adult mice injection of even under 20 μ l of each patient's serum. Due to the limitation of the serum amount, the DIF experiments using patients' serums were performed twice with the similar results for each serum.

Induction of skin inflammation to the mouse ear.

The ears of mice were irritated with either of 0.1 mg/ml PMA in acetone, tape stripping, 0.5% 1-Fluoro-2, 4-dinitrobenzen-fluorodinitrobenze (DNFB) (Nakarai Tesque, Kyoto, Japan) in acetone/olive oil as a hapten, or ultraviolet B (UVB) irradiation. A hapten is applied in non-sensitized mice. In every experiment otherwise indicated, mice were intravenously injected with 10 μ g of AK18, and the ears were collected 24 h later. As for PMA, a hapten, and a tape-stripping, they were applied to the ears of mice simultaneously with intravenous AK18 injection. As for UVB, intravenous AK18 injection was conducted just after the last irradiation to mice. In particular, irradiation was conducted as 120 mJ/cm² for one day (day 0, total 120 mJ/cm²), two days (day -1 and day 0, total 240 mJ/cm²), three days (day -2, day -1, and day 0, total 360 mJ/cm²), or 240 mJ/cm² for three days (day -2, day -1, and day 0, total 720 mJ/cm²) before

intravenous AK18 injection at day 0. The ear swelling levels were measured 24 h after the final UVB irradiation.

Induction of hair loss and DIF analysis in mice with intravenous AK23 injection.

50 µg of AK23 was intravenously injected to mice simultaneously with PMA application to the dorsal skin. The boundary lesion to cover both untreated and PMA-painted areas of the dorsal skin was evaluated for DIF analysis two days after PMA and AK23 administration.

Histamine prick to the mouse ears.

10 mg/ml histamine dihydrochloride (Sigma-Aldrich) in PBS was administered with pricking of the dorsal ear with lancet needles. PBS was also administered as a control. Histamine prick was administered simultaneously with 20 µg of intravenous injection of AK18, and epidermis was collected 24 h later for a flow cytometric analysis.

Statistical analysis.

Unless otherwise indicated, data are presented as means ± SDs and each data point is representative of three independent experiments. Statistical analyses were performed using Graphpad prism (GraphPad Software, Inc., CA), using either of a parametric Student's t-test or one-way ANOVA test. A value of $p < 0.05$ was considered to indicate statistical significance, and indicated as *. NS indicates not significant.

Supplemental Figure legends**Figure E1. A flow cytometry-based analysis for detecting AK18 deposition in the murine epidermis**

A, A schema of preparation of epidermal cell suspension. **B**, Identification of keratinocytes (a) and hematopoietic cells (b). **C**, Expression of Fc γ receptors among keratinocytes, compared to isotype-stained control. **D**, Identification of IgG1 and IgG1-MFI among keratinocytes after injection of PBS, 20 μ g of AK18, or 20 μ g of control IgG1, in PMA-painted or untreated ears.

Figure E2. Human anti-Dsg3 Ab deposition in the untreated and PMA-painted mouse skin

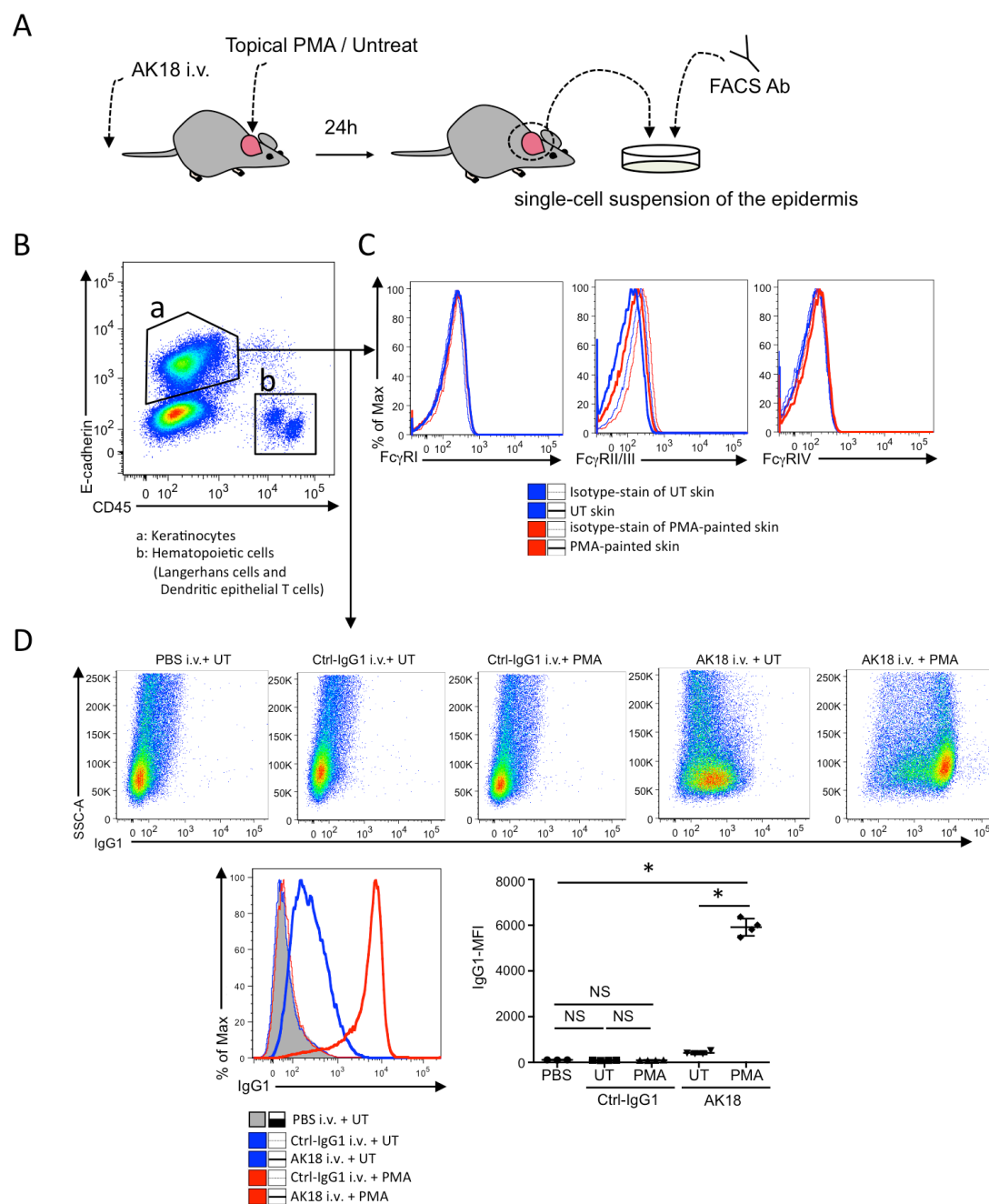
Immunohistochemical evaluation for Ab deposition after intravenous injection of various doses of serum from a pemphigus vulgaris patient (PV) #2 whose anti-Dsg3 Abs were of IgG4 subclass.

Figure E3. Phenotype of a murine pemphigus model

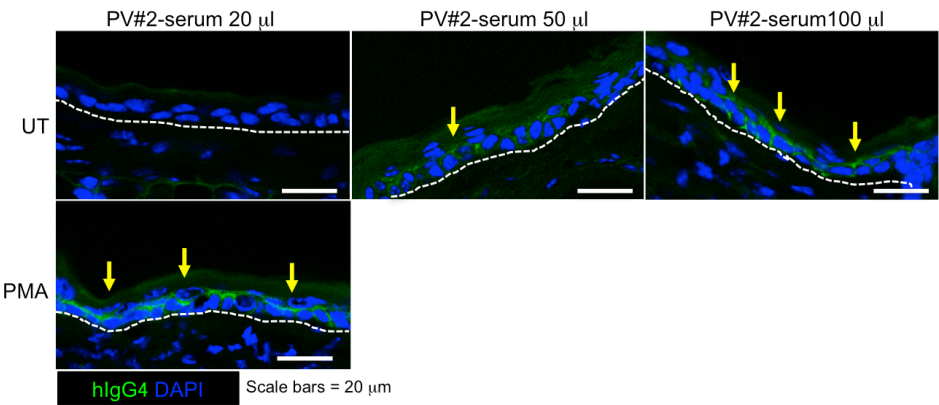
Manifestation of hair loss after intravenous injection of AK23 or AK18. The injection dose is indicated above each mouse.

Figure E4. A conceptual drawing of kinetics of IgGs including anti-Dsg Abs in the skin

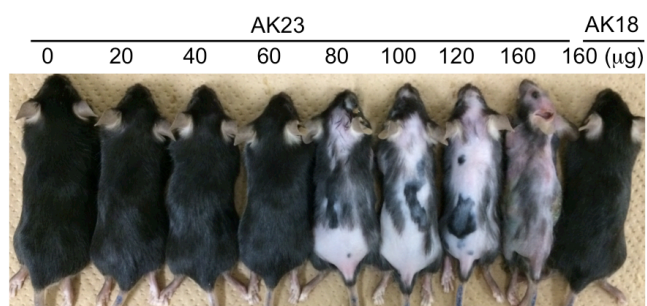
A, No non-specific IgGs deposition in the epidermis. **B**, Low epidermal anti-Dsg Ab deposition and unapparent skin manifestations with low serum Ab titers. **C**, Accumulation of epidermal anti-Dsg Ab deposition and induction of intraepidermal blistering with high serum Ab titers. **D**, Enhancement of epidermal anti-Dsg Ab deposition by local inflammation and induction of intraepidermal blistering with low serum Ab titers.

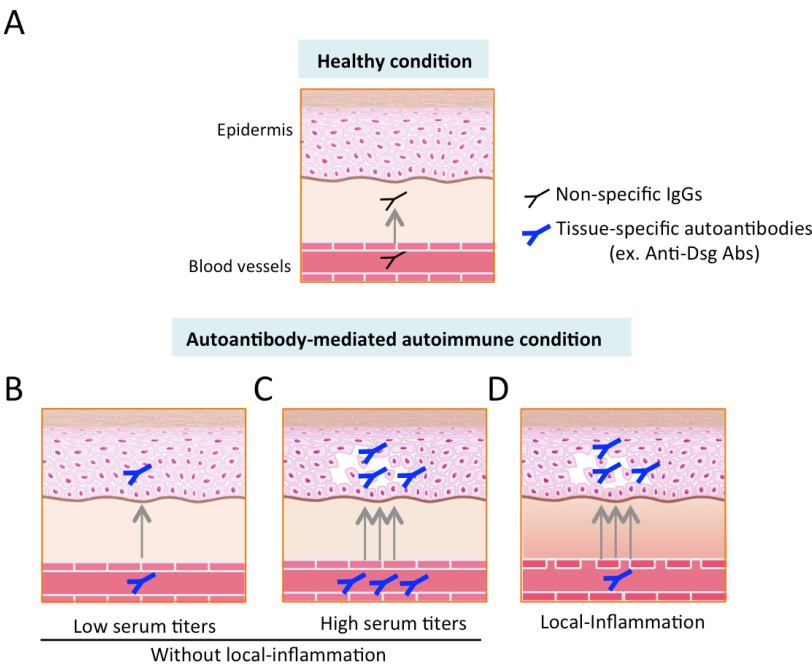


Supplemental Figure E1



Supplemental Figure E2





Supplemental Figure E4