Standardized Production of Anti-Desmoglein 3 Antibody AK23 for Translational Pemphigus Vulgaris Research

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Published in the Pharmacology section

Antibody-mediated receptor activation is successfully used to develop medical treatments. If the activation induces a pathological response, such antibodies are also excellent tools for defining molecular mechanisms of target receptor malfunction and designing rescue therapies. Prominent examples are naturally occurring autoantibodies inducing the severe blistering disease pemphigus vulgaris (PV). In the great majority of patients, the antibodies bind to the adhesion receptor desmoglein 3 (Dsg3) and interfere with cell signaling to provoke severe blistering in the mucous membranes and/or skin. The identification of a comprehensive causative signaling network downstream of antibody-targeted Dsg3 receptors (e.g., shown by pharmacological activators or inhibitors) is currently being discussed as a basis to develop urgently needed first-line treatments for PV patients. Although polyclonal PV IgG antibodies have been used as proof of principle for pathological signal activation, monospecific anti-Dsg3 antibodies are necessary and have been developed to identify pathological Dsg3 receptor–mediated signal transduction. The experimental monospecific PV antibody AK23, produced from hybridoma cells, was extensively tested in our laboratory in both in vitro and in vivo models for PV and proved to recapitulate the clinicopathological features of PV when generated using the standardized production and purification protocols described herein. © 2024 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Bovine IgG stripping from FBS and quality control
Basic Protocol 2: AK23 hybridoma expansion and IgG production
Basic Protocol 3: AK23 IgG purification
Basic Protocol 4: AK23 IgG quality control

CURRENT PROTOCOLS
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Current Protocols e1118, Volume 4
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.1118

Support Protocol 1: Detection of endotoxin levels
Support Protocol 2: Detection and removal of mycoplasma

Keywords: experimental pemphigus vulgaris (PV) antibody AK23 • PV • PV IgG • signal transduction

How to cite this article:
Mueller, E. J., Rahimi, S., Sauta, P., Shojaeian, T., Durrer, L., Quinche, S., Francois, M., Locher, E., Edler, M., Illi, M., Gentinetta, T., Lau, K., Pojer, F., Borradori, L., & Hariton, W. V. J. (2024). Standardized production of anti-desmoglein 3 antibody AK23 for translational pemphigus vulgaris research. *Current Protocols*, *4,* e1118. doi: 10.1002/cpz1.1118

INTRODUCTION

Pemphigus vulgaris (PV) is a potentially life-threatening autoimmune disease of the skin and mucous membranes (Di Zenzo et al., 2016; Hammers & Stanley, 2016). In the majority of PV patients, autoantibodies are directed against the desmosomal cadherin Dsg3 (mucosal PV, Dsg3) or Dsg3 and Dsg1 (mucocutaneous PV). Desmosomal cadherins are intercellular adhesion molecules assembling into multimeric protein complexes called desmosomes that confer strong cell–cell adhesion in tissues subjected to substantial mechanical strain such as the skin and the heart (Getsios et al., 2004; Green et al., 2020). Biochemical fractionation and pharmacological interventions as well as electron microscopical analyses have shown that pathogenic anti-Dsg3 antibodies preferentially bind to extradesmosomal Dsg3 receptors (outside of desmosomes) in cultured keratinocytes to trigger loss of intercellular adhesion through cellular signaling (Di Zenzo et al., 2016; Sato et al., 2000; Rahimi et al., unpublished data). Signal induction occurs through uncoupling of *trans*-adhering Dsg3 between neighboring cells (Heupel et al., 2008), a process involving receptor signaling (Di Zenzo et al., 2016; Mueller et al., 2008; Spindler et al., 2023) and likely mechanosensing and mechanosignaling (Rubsam et al., 2017).

Current treatment options for PV patients comprise immune-suppressive medications and various other biological drugs including anti-CD20 antibodies (Rituximab). These measures take at least 3 to 4 months to become effective in the patient (Joly et al., 2017; Werth et al., 2021). A first-line treatment to prevent blistering and speed up the healing process is thus needed, preferentially aiming to control the antibody-mediated Dsg3 signaling response in targeted keratinocytes and support healing of existing blisters. A variety of deregulated, causative signals have been identified in both in vitro and in vivo PV models (Rahimi et al., unpublished data). Nonetheless, the current understanding of a comprehensive signaling network governing PV pathophysiology, downstream of antibody-mediated Dsg3 receptor signaling, is still unclear. To move forward with a therapeutic signaling strategy, the highly modulated, longitudinal autoantibody-induced signaling network needs to be understood in detail. To this end, experimentally produced mono- or bi-specific antibodies targeting Dsg3 or Dsg3 and Dsg1 such as AK23, PX43, or 2G4 are of considerable interest to support a viable therapeutic strategy, while recapitulating PV blistering. Among the monospecific Dsg3 antibodies, AK23, produced in an active transfer mouse model (Tsunoda et al., 2003), has been most widely used by several laboratories including ours to study PV pathophysiology under various experimental conditions (Rahimi et al., unpublished data).

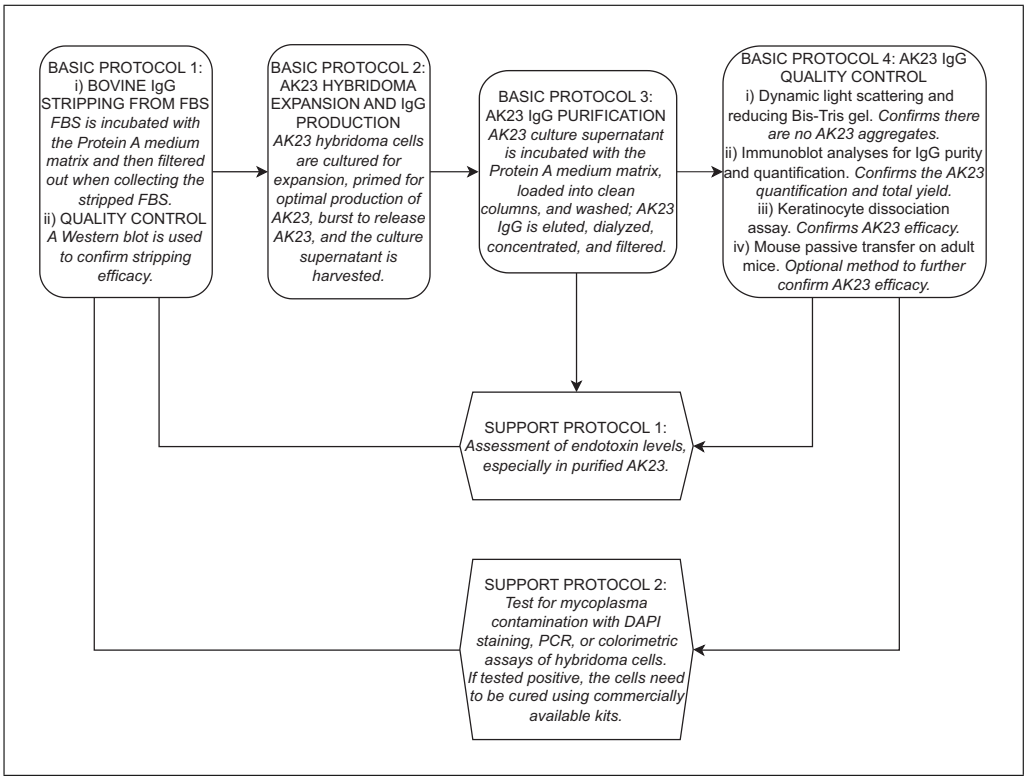

Figure 1 Overview of the stages of AK23 IgG production, with references to the Basic and Support Protocols and a description of the main steps.

For the past nearly two decades, the production of AK23 from hybridoma cells by various labs in the PV community was a challenging endeavor due to unknown factors at the time, greatly affecting the effectiveness and reproducibility of AK23. Considering the importance of the research on Dsg3 and that AK23 is an invaluable antibody to specifically study Dsg3 mechano- and biochemical signaling, such unknown factors may delay and sometimes even falsify crucial research. Therefore, our lab, in past and current collaborations with CSL Behring (Williamson et al., 2006) and EPFL Lausanne, optimized the steps for the production and quality control of AK23, ensuring its reproducible activity. The three biggest challenges we resolved in this process were as follows: i) recognizing and clearing myoplasm infection of the original AK23 hybridoma stock, ii) clearing bovine IgG contaminations in fetal bovine serum (FBS) as it preferentially affinity purifies due to higher avidity for protein A than AK23, and iii) preventing and monitoring endotoxin contamination. In this article, we share our expertise in producing AK23 to improve the quality and reproducibility of this invaluable tool for research related to Dsg3 receptor signaling and PV.

We provide the protocols to produce the monoclonal antibody AK23, with reproducible activity, from the AK23 hybridoma cells (Tsunoda et al., 2003) as follows: bovine IgG stripping from FBS and quality control (Basic Protocol 1), AK23 hybridoma expansion and IgG production (Basic Protocol 2), AK23 IgG purification (Basic Protocol 3), AK23 IgG quality control by i) dynamic light scattering (DLS) and reducing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), ii) immunoblotting, iii) keratinocyte dissociation assay (KDA), and iv) passive transfer of AK23 into mice (Basic Protocol 4). Further methods to successfully test AK23 batches are as follows: detection of endotoxin levels (Support Protocol 1), and detection and removal of mycoplasma (Support Protocol 2). An overview of the stages of AK23 IgG production with references to the Basic and Support Protocols and a description of the main steps is shown in Figure 1.

NOTE: For safety reasons, practicing aseptic techniques, wearing a lab coat and gloves, and using a sterile laminar flow hood are mandatory in the cell culture lab.

NOTE: To avoid contamination, all solutions and equipment coming into contact with living cells must be sterile, and proper aseptic techniques must be used.

NOTE: To prevent endotoxin contaminations, all reusable devices, columns, pumps, and membranes must be thoroughly cleaned with 0.5 M NaOH and rinsed with distilled water. Solutions are produced with endotoxin-free components.

NOTE: All cell cultures were performed in a 37°C, 5% CO_2, and 90% humidity cell incubator unless otherwise specified.

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals. Appropriate informed consent is necessary for obtaining and using human study material.

BASIC PROTOCOL 1

BOVINE IgG STRIPPING FROM FBS AND QUALITY CONTROL

The protocols below describe the i) stripping of bovine IgG from FBS and ii) quality control of the stripped FBS prior to starting the AK23 hybridoma cell culture.

Note that bovine IgG–free FBS can be purchased but might, as in our case, not support optimal hybridoma growth [1 population doubling (PD)/1.5 days]. Several FBS providers or lots will need to be tested alongside each other and compared for optimal growth before starting AK23 production. Successful stripping of bovine IgG is confirmed through immunoblotting.

i) Bovine IgG stripping from FBS

Materials

0.5 M NaOH
MabSelect Protein A medium matrix (Amersham BioScience, cat. no. 17-5199-03 or equivalent), 0.5 M NaOH- treated (endotoxin-free)
If using a matrix other than MabSelect Protein A, make sure it is NaOH-treated.
Phosphate buffered saline (PBS, see recipe or equivalent)
FBS (GIBCO, cat. no. 10270, lot no. 41G7642K, Gamma globulin 126.86 µg/mL)

Endotoxin-free glassware (cleaned, autoclaved, and 0.5 M NaOH-treated)
George Roller Bottle, 2 L, GL45 Screw Neck, 3.3 Borosilicate Glass, 10/CS (cleaned, autoclaved, and 0.5 M NaOH- treated; Foxx Life Sciences, cat. no. 1600030-FLS or equivalent)
WHEATON Roller System (Duran Wheaton Kimble or equivalent)
High-throughput bottle-top filter, 0.2 µm (Thermo Fisher Scientific, cat. no. 597-4520 or equivalent)
Laminar flow hood (Labconco Purifier BSC Class II or equivalent)
Poly-Prep chromatography columns (disposable polypropylene columns; 2 mL bed volume and 10 mL reservoir volume; Biorad, cat. no. 7311550 or equivalent)

1. To avoid endotoxin contamination, rinse all glassware and columns for a minimum of 30 min with 1× device volume of 0.5 M NaOH.
2. Thoroughly rinse them with distilled water to remove remaining NaOH.
3. Centrifuge 1 volume (10 mL required for 1 L FBS) MabSelect Protein A medium matrix for 5 min at 300 × *g* at room temperature. Discard supernatant and incubate the precipitate with 1 volume of 0.5 M NaOH for 30 min to strip from endotoxin. Repeat

the centrifugation and aspiration steps and then rinse with 3 volumes of distilled water. Repeat the centrifugation and aspiration steps again and then resuspend in 1 volume PBS.

4. Pour FBS in 2-L roller bottles and add 10 mL endotoxin-free MabSelect Protein A medium matrix to every 1 L of FBS.

5. Mix overnight on the roller system at 4°C.

   Bovine IgG will be stripped from FBS by binding to the Protein A resin.

6. Load the Protein A mixture into an empty 2-mL chromatography column according to the manufacturer's guidelines and collect the flow-through consisting of IgG-stripped FBS.

   Use a fresh column when the flow of FBS stops.

7. Filter FBS with the 0.2-µm high-throughput bottle-top filter in the laminar flow hood.

8. Use IgG-stripped FBS immediately; either store it at 4°C for a few days or freeze at −20°C for long-term storage.

   Set aside an aliquot of 10 µL for quality control (Basic Protocol 1 ii).

ii) Quality control of FBS after stripping from bovine IgG

Materials

Western Blot gel casting, gel running, and protein transfer devices
mPAGE TurboMix Bis-Tris Gel Casting Kit (Merck, cat. no. TMKIT-60 or equivalent)
Transfer buffer powder for use with mPAGE Bis-Tris gels (Merck, cat. no. MPTRB or equivalent)
MOPS SDS running buffer powder for mPAGE Bis-Tris gels (Merck, cat. no. MPM0PS or equivalent)
4× mPAGE LDS sample buffer (Merck, cat. no. MPSB-10ML or equivalent)
Prestained protein ladder (Thermo Fisher Scientific, cat. no. 26619 or equivalent)
Lyophilized skimmed milk (any commercial source)
Tris buffered saline (TBS) [500 mL 1 M TRIS, pH 7.5 and 45 g NaCl (Fisher Chemical or equivalent) in 4500 mL distilled water)]
TBST (TBS with 0.05% Tween 20)
Tween 20 (Merck; cat. no. P1379 or equivalent)
Rabbit anti-bovine antibody (Bethyl Laboratories, cat. no. A10-102A or equivalent)
Anti-rabbit antibody (LI-COR, cat. no. 926-32213 or equivalent)
10% SDS (Merck or equivalent)
TBST with 0.1% SDS (for secondary antibody)
10% ammonium persulphate (APS; aliquots stored at −20°C, Merck or equivalent)
Tetramethylethylenediamine (TEMED; AppliChem or equivalent)

Gel loading tips (Thermo Fisher Scientific or equivalent)
Polyvinylidene fluoride (PVDF) membrane (Merck or equivalent)
Orbital shaker (Rotamax 120, P/N: 544-41200-00; Heidolph or equivalent)
Odyssey imaging system (LI-COR, cat. no. 9120 or equivalent)

1. Perform a reducing SDS-PAGE according to the Merck mPAGE protocol to assess efficacy of bovine IgG stripping from FBS.

   A 4% to 12% reducing Bis-Tris gel leads to a good resolution and clear bands. Load 15% diluted FBS and 15% diluted stripped FBS (from i)) with 1× mPAGE LDS sample buffer. The volume depends on the number of wells and the thickness of your gel. Typically, we

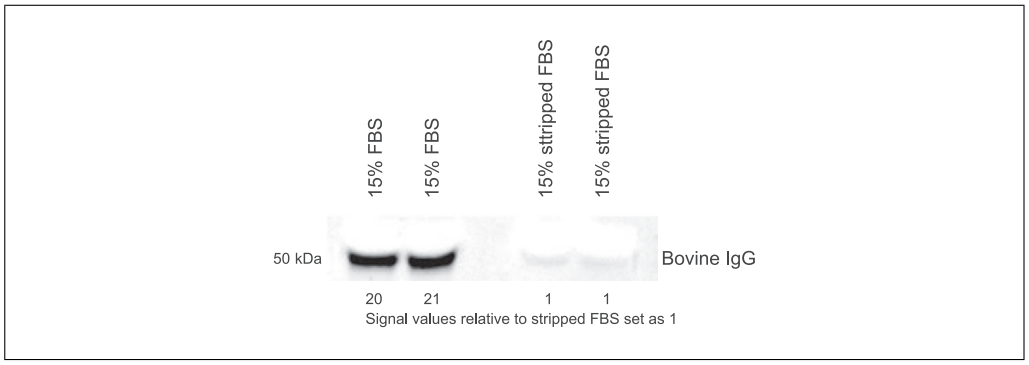

Figure 2 Bovine IgG stripping quality control by Western blotting followed by immunoblot analyses of bovine IgG. The signal values are relative to the stripped FBS. A clear reduction (~95%) in bovine IgG is visible between non-stripped and stripped FBS.

load 15 µL per well on a 15-well, 1-mm-thick gel. It is recommended to dilute the FBS to 15% as non-diluted FBS has high amounts of albumin, which would skew the bovine IgG, making it less reliable for quantification.

2. Transfer proteins to the PVDF membrane.
3. Block the membrane in 5% skimmed milk in TBST for 1 h at room temperature with gentle mixing on the orbital shaker.
4. Incubate the membrane overnight at 4°C for 1 h at room temperature with rabbit anti-bovine antibody (1/5,000) in 5% skimmed milk in TBST with gentle mixing on the orbital shaker.
5. Wash the membrane 3 × for 5 min in TBST with gentle mixing on the orbital shaker.
6. Incubate with anti-rabbit antibody (1/10,000) in 5% skimmed milk in TBST buffer containing 0.1% SDS for 1.5 h at room temperature with gentle mixing on the orbital shaker.
7. Wash the membrane 2 × for 5 min in TBST and then for 5 min in TBS with gentle mixing on the orbital shaker.
8. Scan the membrane using the Odyssey imaging system to reveal the bound antibodies.

   Good quality stripping of FBS should lead to ~95% reduction in bovine IgG (Fig. 2).

9. Confirm if the FBS is endotoxin-free (see Support Protocol 1).

BASIC PROTOCOL 2

AK23 HYBRIDOMA EXPANSION AND IgG PRODUCTION

The protocol below describes the culture of AK23 hybridoma cells for IgG production and harvest of IgG-containing cell culture supernatant.

Materials

AK23 hybridoma cells (Tsunoda et al., 2003)
The AK23 hybridoma cells were a kind gift from Prof. Masayuki Amagai (Department of Dermatology, Keio University School of Medicine, Tokyo, Japan).
Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, cat. no. 22400-089 or equivalent)
Do not add antibiotics and antimycotics to the culture medium as these compounds may interfere with hybridoma productivity.
FBS (GIBCO, cat. no. 10270, lot no. 41G7642K; gamma globulin 126.86 µg/mL)
Ethanol, 70%

IgG-stripped FBS (see Basic Protocol 1)
PBS (see recipe or equivalent)

T75 cell culture flasks (TPP, cat. no. 9007 or equivalent)
Cell culture water bath
Laminar flow hood (Labconco Purifier BSC Class II or equivalent)
Cell incubator, 37°C, 5% CO_2, and 90% humidity (Memmert GmbH, Type INC0153med or equivalent)
Cell culture microscope (Nikon or equivalent)
Sterile polypropylene centrifuge tubes, 50 mL (TPP, cat. no. Z707708 or equivalent)
Swing out centrifuge (Eppendorf, cat. no. 5810R or equivalent)
Hemacytometer with trypan blue or equivalent cell counter
Cell culture camera (Nikon or equivalent)

1. Thaw the number of vials of AK23 hybridoma cells required to obtain the desired amount of AK23 (Table 1) and add 1×10^6 cells to 20 mL prewarmed RPMI/20% non-stripped FBS per T75 flask.

   Heat the water bath to 37°C before starting the cell culture. One vial of cells at a time is swirled in the water bath at 37°C such that the frozen sample is fully immersed. When there is only a small frozen pellet left, clean the outside of the cryotube with 70% ethanol and transfer it to the laminar flow hood.

2. Flasks containing thawed hybridoma cells are transferred to the cell culture incubator.

   Flasks must be kept upright for optimal cell expansion.

   Cells must be regularly checked under the light microscope for growth, morphology, and fitness.

3. After 3 days of culture, add another 10 mL RPMI/20% non-stripped FBS per T75 flask and culture for another 2 days.

4. After 2 days, transfer the medium containing cells ($\sim 10^7$ cells/30 mL per flask) into a 50-mL centrifuge tube and centrifuge for 5 min at $300 \times g$ at room temperature.

5. Discard supernatant and for each starting (from step 4) 30 mL of culture supernatant, resuspend cells in 1 mL ($\sim 10 \times 10^6$ cells/mL) prewarmed RPMI/15% stripped FBS (see Basic Protocol 1). Count the cells in the hemacytometer using trypan blue (to identify dead cells).

   Note that the hybridoma cells grow in grape-like clusters (Fig. 3) and therefore automatic counting is error-prone.

6. If necessary, repeat steps 1 to 5 to obtain enough cells to produce the desired amount of AK23 IgG in steps 7 to 12 (Table 1).

Table 1 Overview of Cells and Media Required for AK23 Hybridoma Expansion and IgG Production

		Steps 1–6			Steps 7–12		
AK23 produced	AK23 produced (5 mg/mL)	Cells seeded	T75 flasks (1×10^6 cells/flask)	Total culture medium (L)	Cells seeded	T75 flasks (1×10^6 cells/flask)	Total culture supernatant (L)
~15 mg	2 mL	3.33×10^6	3	0.1	3.33×10^7	33	1
~150 mg	20 mL	3.33×10^7	33	1	3.33×10^8	333	10

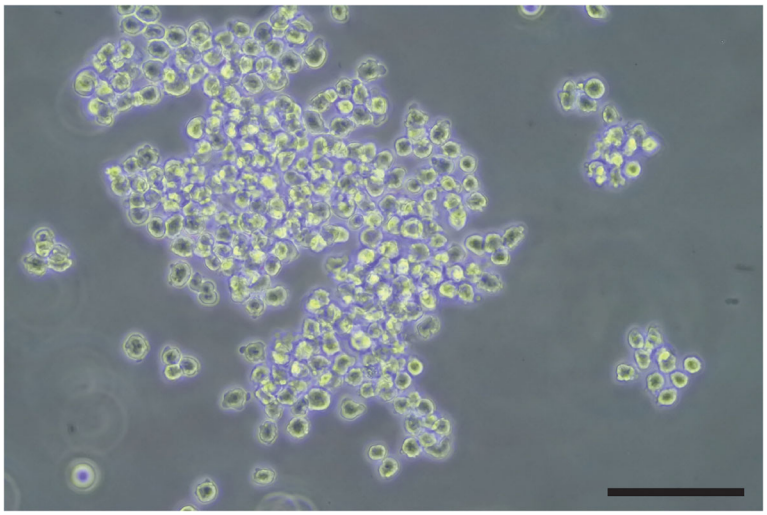

Figure 3 Healthy AK23 hybridoma cells grow in grape-like clusters. Scale bar: 100 µm.

Note that optimal cell growth (with >90% viability) is around 1 PD per 1.5 days. PD = 3.33 Log10 (Viable cells / Seeded cells). Surplus hybridoma cells from steps 1 to 6 are frozen for subsequent AK23 production using standard protocols.

7. Inoculate 1×10^6 cells/flask in 20 mL prewarmed RPMI/15% stripped FBS (see Basic Protocol 1). Calculate the required number of cells and T75 flasks to obtain the desired amount of AK23 from Table 1.

   To produce 150 mg AK23, 3.33×10^7 cells are seeded in step 1 in 33 T75 flasks and 3.33×10^8 cells are required in step 7 to inoculate 333 T75 flasks and harvest 10 L culture supernatant. On average, 15 mg AK23 IgG per liter supernatant is harvested (Table 1). Instead of T75, T150 flasks or, if available, bioreactor-type devices are also suitable.

8. After 2 days, add 10 mL RPMI/15% stripped FBS (see Basic Protocol 1). Continue culture for no longer than 2 to 3 days.

9. Swirl the flask and then transfer the 30 mL supernatant and all cells (approximately 1×10^7 cells/flask) into 50-mL centrifuge tubes (or larger tubes if available).

10. Centrifuge at 1,800 × *g* for 10 min at room temperature. The high speed serves to break hybridoma cells, which release the IgGs. Collect the supernatant and wash the cell pellets twice with 1 mL PBS.

11. Pour the supernatant into the sterile, empty RPMI media bottles (which are endotoxin-free) and combine with PBS, after centrifugation at 1,800 × *g*.

    Keep a 500-µL aliquot for quantification of IgG in the supernatant before IgG purification (∼15 mg/L) to validate the efficacy of production and recovery (Fig. 4).

12. Immediately process the supernatant for IgG purification (Basic Protocol 3) or store at 4°C for no longer than 3 days.

    We typically process ∼10 L of IgG-containing culture supernatant per experiment.

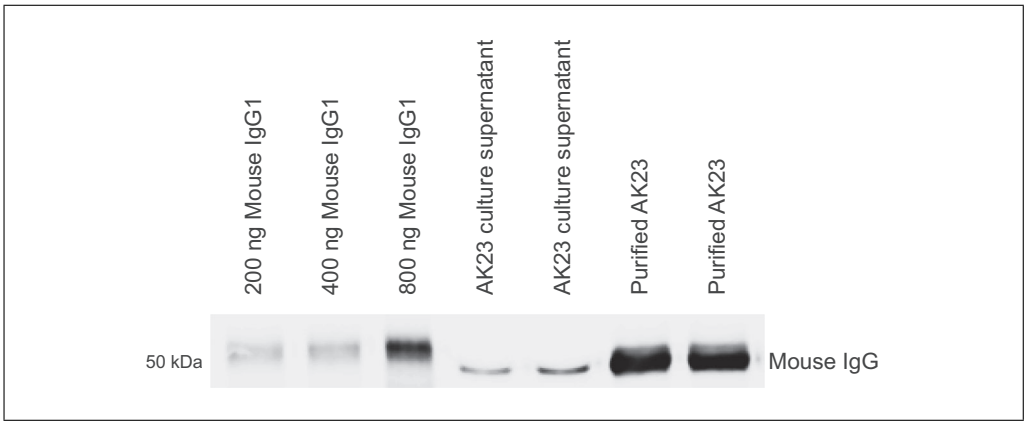

Figure 4 AK23 quality control by Western blotting followed by immunoblot analyses of mouse IgG. The mouse IgG1 standards are used to plot a curve and quantify the amount of AK23 (mouse IgG) in the AK23 supernatant and purified AK23.

AK23 IgG PURIFICATION

BASIC PROTOCOL 3

The protocol below describes the isolation of AK23 IgG from the culture supernatant (see Basic Protocol 2). It typically allows recovery between 60% and 90% of IgG (~15 mg/L medium supernatant).

Materials

AK23 culture supernatant (from Basic Protocol 2)
0.5 M NaOH
0.5 M NaOH-treated endotoxin-free MabSelect Protein A medium matrix (Amersham BioScience, cat. no. 17-5199-03 or equivalent) (see Basic Protocol 1)
0.1 M glycine, pH 3.0
1.5 M Tris, pH 8.0
PBS + 3 mM NaOAc, pH 7.4
PBS (see recipe or equivalent)

0.5 M NaOH-treated endotoxin-free glassware and devices (see Basic Protocol 1)
George Roller Bottle, 2 L, GL45 Screw Neck, 3.3 Borosilicate Glass, 10/CS (cleaned, autoclaved and 0.5 M NaOH-treated) (Foxx Life Sciences, cat. no. 1600030-FLS or equivalent)
WHEATON Roller System (Duran Wheaton Kimble or equivalent)
Econo-Pac chromatography columns (disposable, empty polypropylene columns, 0 to 20 mL bed volume; Biorad, cat. no. 7321010 or equivalent)
Spectra/Por 1 dialysis membrane, 12 to 14 kDa (Carl Roth, cat. no. 1964.1 or equivalent)
Filter, 0.2 µm (Thermo Fisher Scientific, cat. no. 723-2520 or equivalent; for 10 to 100 mL samples)
Laminar flow hood (Labconco Purifier BSC Class II or equivalent)
Amicon Ultra-15 centrifugal filters, 100 kDa (Merck, cat. no. C7715 or equivalent)

1. Pour the supernatant from Basic Protocol 2 into endotoxin-free 2-L roller bottles. Add 10 mL MabSelect Protein A medium matrix to every 1 L of culture supernatant and mix overnight on the roller system at 4°C.

   This will allow AK23 IgG to bind efficiently to the Protein A medium matrix.

2. Per disposable column, load 2 L culture supernatant (20 mL MabSelect Protein A medium matrix) with bound AK23 IgG and wash thoroughly with 20 column volumes of PBS.

Typically, 5 disposable columns and 2 L PBS will be used to load and wash 100 mL MabSelect Protein A matrix collected from 10 L of supernatant.

3. Elute AK23 IgG with 5 column volumes of glycine (0.1 M, pH 3.0) and immediately neutralize the eluted fractions with 1.2 column volumes of 1.5 M Tris (pH 8.0).

   To avoid damaging the IgG protein under low pH, let it elute and directly drop into a device containing the necessary volume of 1.5 M Tris (pH 8.0).

4. Use the Spectra/Por 1 (12 to 14 kDa) dialysis membrane to dialyze the neutralized eluted fractions against PBS supplemented with 3 mM NaOAc (pH 7.4) overnight at 4°C.

   To avoid IgG precipitation, refrain from dialyzing against PBS alone.

5. Recover the dialyzed AK23 sample and concentrate with Amicon Ultra-15 Centrifugal Filter (100 kDa) by centrifugation for 15 min at 3,000 × *g*. The final concentration should be around 1 mg/mL for cell culture and 5 mg/mL for passive transfer into mice (see Basic Protocol 4).

   The IgG concentration is measured using the optical density at 280 nm (OD280; 1.37 OD = 1 mg/mL). In total, roughly 2 mL and 20 mL AK23 at a concentration of 5 mg/mL are recovered from 1 L and 10 L culture supernatant, respectively (Table 1).

6. Filter and sterilize the dialyzed AK23 IgG with a 0.2-µm filter in a laminar flow hood; aliquot and keep at 4°C for short-term storage and at −20°C for long-term storage.

   This step is mandatory to prevent IgG degradation, cell culture contamination, and mice sepsis.

7. Confirm if the AK23 is endotoxin-free (see Support Protocol 1).

   This step is mandatory to avoid affecting the health of mice and preventing non-specific signaling (such as an inflammatory response).

BASIC PROTOCOL 4

AK23 IGG QUALITY CONTROL

Once endotoxin-free AK23 production has been confirmed, quality control of AK23 IgG is done using three mandatory steps and one optional step, as described in the following protocols: i) purity and integrity: DLS and reducing SDS-PAGE, ii) IgG purity and quantification: immunoblot analyses, iii) AK23 efficacy tested on cultured keratinocytes: KDA, and iv) AK23 efficacy tested in vivo: mouse passive transfer (optional).

i) Purity and integrity: DLS and reducing SDS-PAGE

Materials

Purified AK23 (from Basic Protocols 2 and 3)
Reducing SDS-PAGE/immunoblot materials (see Basic Protocol 1 ii)
Coomassie blue dye stain materials

Stunner DLS analyzer and 96-well Stunner plates (Unchained Labs or equivalent)
Camera to capture a picture of the SDS-PAGE gel

1. Load 2 µL, in duplicates, of each sample and buffer onto the Stunner DLS plates and collect data using standard settings (Fig. 5A and 5B).

   Note that the DLS analysis displays the mass and intensity distributions of the purified AK23. A single peak is expected at ∼10 nm corresponding to the IgG and no other peaks above ∼10 nm should be present to confirm lack of aggregates.

2. Perform a reducing SDS-PAGE according to the Merck mPAGE protocol to assess efficacy of AK23 purification.

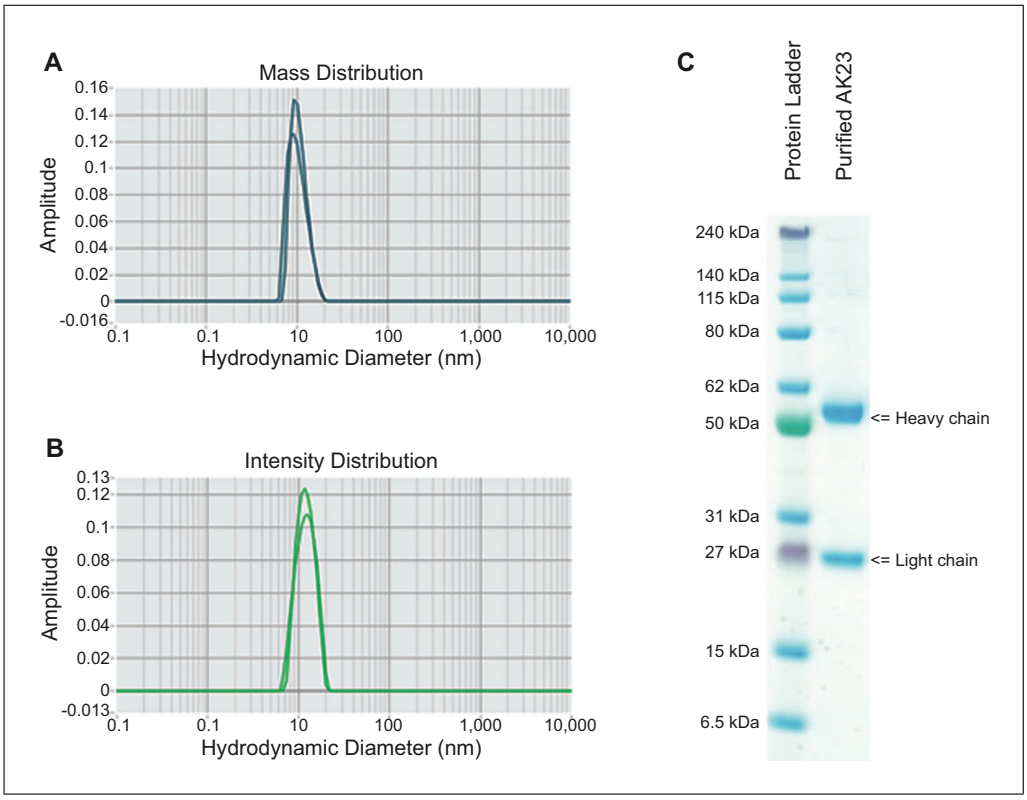

Figure 5 Dynamic light scattering analysis in duplicates displaying the mass distribution (**A**) and intensity distribution (**B**) of the purified AK23. The peaks at ~10 nm correspond to the expected value for IgG and absence of other peaks above ~10 nm means there are no aggregates. (**C**) The corresponding reducing Western blot gel for the purified AK23 reveals two bands, displaying heavy (50 kDa) and light (25 kDa) chains with no signs of aggregates.

Using 4% to 12% Bis-Tris gel leads to good resolution of the IgG heavy and light chains (25 and 50 kDa; Fig. 5C) and concise bands. Load a protein ladder and 1 µg purified AK23.

3. When the gel electrophoresis is finished, do a standard Coomassie blue dye stain until clear bands are visible against the background.
4. Picture the blue stained gel (Fig. 5C).

Note that the blue stain is expected to reveal only two bands in the purified AK23, representing the heavy (50 kDa) and light (25 kDa) chains with no signs of aggregates.

ii) Immunoblot analyses for IgG identification and quantification

Materials

Purified AK23 (from Basic Protocol 2 and 3)
Western blot materials (see Basic Protocol 1 ii)
Isotype control mouse IgG1 (Bio X Cell, cat. no. BP0083 or equivalent)
BSA standard (Roche, cat. no. 10735094001 or equivalent)
Anti-mouse antibody (LI-COR, cat. no.926-68072 or equivalent)

PVDF membrane (Merck or equivalent)
Orbital shaker (Rotamax 120, P/N: 544-41200-00; Heidolph or equivalent)
Odyssey imaging system (LI-COR, cat. no. 9120 or equivalent)

1. Perform a Western blot (see Basic Protocol 1 ii)) to identify and quantify AK23.

Using 4% to 12% Bis-Tris gel leads to a good resolution and clear bands. It is recommended to load at least three different concentrations of isotype control mouse IgG1 to

form a standard curve. The range is based on the previously measured OD280 of the purified AK23 (1.37 OD = 1 mg/mL). Load a protein ladder in unicate, and AK23 culture supernatant and purified AK23 in duplicates.

2. Transfer proteins to the PVDF membrane.
3. Block the membrane in 5% BSA in TBST for 1 h at room temperature with gentle mixing on the orbital shaker.
4. Incubate the membrane with anti-mouse antibody (1/10,000) in 5% BSA in TBST buffer containing 0.1% SDS and 0.1% Tween 20 for 1.5 h at room temperature with gentle mixing.
5. Wash the membrane twice for 5 min in TBST with gentle mixing and then for 5 min in TBS with gentle mixing.
6. Use the Odyssey imaging system to scan the membrane to detect the bound antibodies.
7. Using the signals of the isotype control mouse IgG1 concentration standards, plot a standard curve to determine the amount of purified AK23. This quantification should match the previously measured OD280 of the purified AK23 (1.37 OD = 1 mg/mL).

   A good quality production should have a yield >10 mg IgG per liter of AK23 culture supernatant.

   A representative overview of the AK23 quality control immunoblot is shown in Figure 4.

iii) Keratinocyte dissociation assay

The protocol below describes a semiquantitative method to define the effectiveness of AK23 in disrupting Dsg3 *trans*-adhesion using KDA (Caldelari et al., 2001; de Bruin et al., 2007). Hartman et al. (2003) provide an overview of the different steps of KDA.

Materials

Primary human epidermal keratinocyte progenitor (HPEK) cells (CELLnTEC, cat. no. CnT-HPEKp or equivalent)
CnT-07 epithelial proliferation medium (CELLnTEC, cat. no. CnT-07 or equivalent)
Accutase, enzymatic solution for cell passaging (CELLnTEC, cat. no. CnT-Accutase-100 or equivalent)
$CaCl_2$ (Merck, cat. no. C4901 or equivalent)
It is prepared into a 226 mM $CaCl_2$ solution in distilled water, sterilized through a 0.2-µm filter, and stored at 4°C.
Isotype control mouse IgG1 (<0.4 endotoxin units/mg; Bio X Cell, cat. no. BP0083 or equivalent)
Purified AK23 (from Basic Protocols 2 and 3)
Dispase II (neutral protease grade II, Roche, cat. no. 04 942 078 001 or equivalent)
PBS+ (see recipe)
0.1% BSA in distilled H_2O (Merck, cat. no. A2153 or equivalent)
4% formaldehyde (Merck, cat. no. 1.00496 or equivalent)
0.1% crystal violet, diluted in water from a 1% stock (powder; Merck, cat. no. C3886 or equivalent)

Filter, 0.2 µm (Thermo Fisher Scientific, cat. no. 723-2520 or equivalent)
T75 culture flasks (TPP, cat. no. TPP 90076 or equivalent)
24-well plates (TPP, cat. no. TPP 92024 or equivalent)

Cell incubator, 37°C, 5% CO_2, and 90% humidity (Memmert GmbH, Type INC0153med or equivalent)
Laminar flow hood (Labconco Purifier BSC Class II or equivalent)
Barrier pipette tips (Thermo Fisher Scientific, cat. no. 2179-HR or equivalent)
Parafilm
Cell culture microscope (Nikon or equivalent)
Fiji ImageJ version 2.9.0/1.53t (NIH, USA or equivalent)

1. Thaw and seed 4000 primary HPEK cells per cm^2 in a T75 flask (300,000 cells per T75) in the CnT-07 epithelial proliferation medium. The cells are expanded in the cell incubator at 37°C to 80% confluency (~3,000,000 cells per T75) for 1 passage after thawing before entering the KDA experiment.
2. Passage the cells using Accutase according to the manufacturer's protocol.
3. Seed ~300,000 cells in 1 mL of CnT-07 into each well of a 24-well plate (~161,000 cells per cm^2) in triplicates.
4. When the cells are 100% confluent (the next day), add 5 µL 226 mM $CaCl_2$ per well (1 mL) to reach a final concentration of 1.2mM $CaCl_2$.

   CnT-07 contains 0.07 mM $CaCl_2$ and hence an additional 1.13 mM needs to be added.

5. After 6 h, add the isotype control mouse IgG1 or AK23 (an optimal dose is typically 20 µg/mL) to the medium covering the cells and manually shake the plate gently for 5 s.

   Perform an AK23 dose response in the range of 1 to 100 µg/mL and match isotype control mouse IgG1 to determine the optimal dose.

6. Incubate for 24 h in the cell incubator at 37°C.
7. Discard the cell culture medium and add 400 µL of 5 mg/mL Dispase II in PBS+ per well for 25 min in a cell incubator at 37°C.

   Make sure the cell sheet is completely detached; if not, incubate for an additional 25 min at 37°C.

8. Place 1000-µL tips (1 per well) upright in 0.1% BSA no higher than the 500-µL level for 30 min at room temperature. Pipette out the BSA solution from the tips just before use.
9. Apply mechanical stress to the sheet by pipetting exactly 10 times up and down with a 1-mL pipette set to 250 µL and mounted with a BSA-coated tip. To do so, tilt the plate in your direction, place the tip parallel to the well wall, and slowly pipette up and down, each time sucking the cell sheet into the tip.
10. Add 400 µL 4% formaldehyde per well.
11. Add 4.5 µL 0.1% crystal violet per well.
12. Cover the plate with a lid and seal with parafilm to prevent evaporation. Keep the plate at room temperature. For long-term use, the plate may be stored at 4°C.
13. After incubation for at least 24 h, take a picture of each well at a perpendicular angle under well-lit conditions.
14. Using the ImageJ software, import the pictures, set the scale based on your image, and count the fragments larger than 0.0125 mm^2.

    A representative overview of the AK23 quality control and dose response by KDA on HPEK cells is shown in Figure 6.

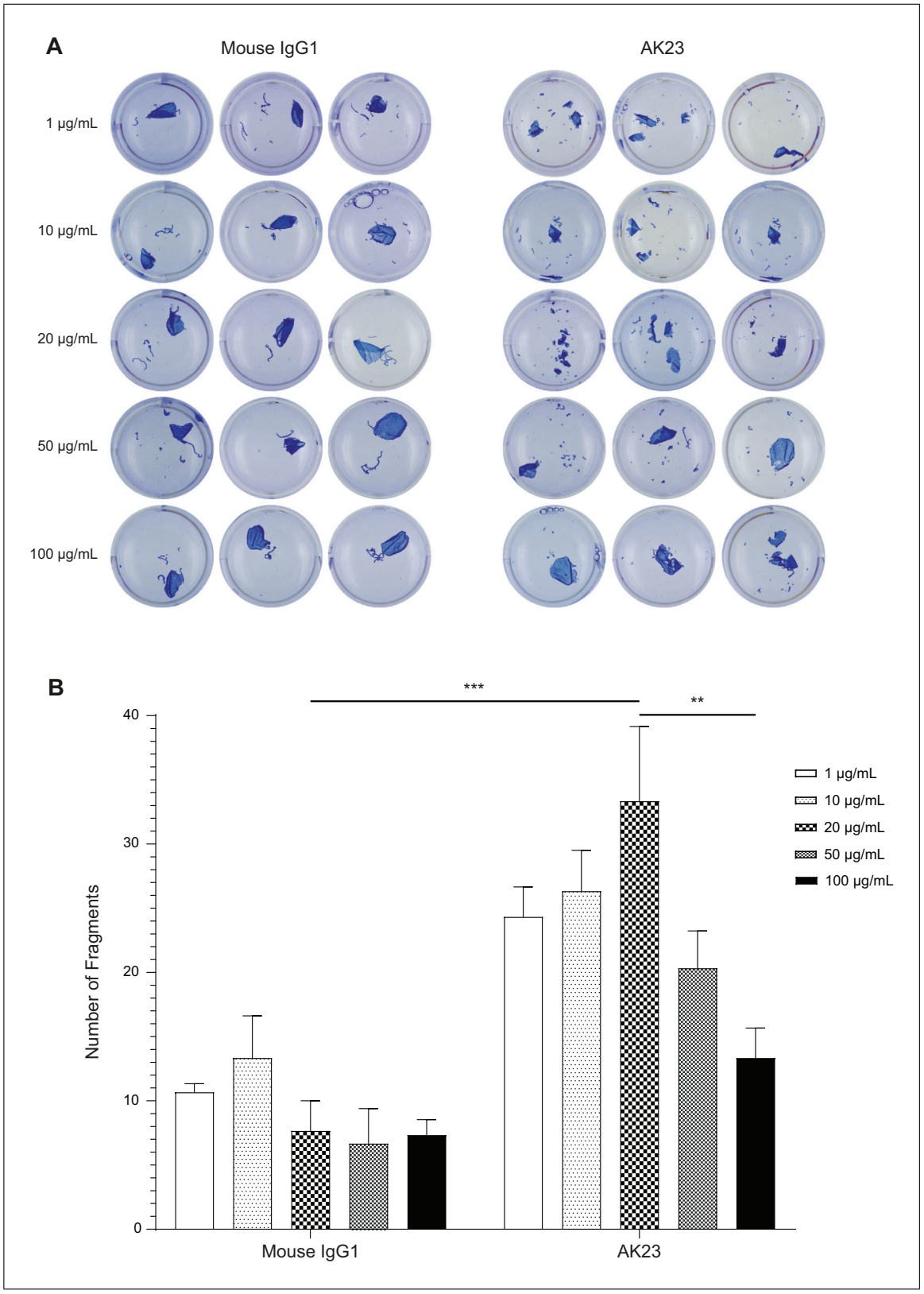

Figure 6 AK23 quality control dose response by keratinocyte dissociation assay on HPEK cells. (**A**) Representative pictures of cell sheets treated with different doses of AK23 or control antibody. (**B**) Quantification of fragments with the highest efficacy of AK23 at 20 µg/mL (>30 fragments). *Note the "hook effect"(Tate & Ward, 2004) when the IgG concentration reaches a too high value (50 µg/mL), leading to a decrease in efficacy*. Per group, n = 3, shown as mean ± standard error of the mean. Data analyzed by two-way analysis of variance with asterisks representing the following *p*-values: $^{**}p < 0.01$ and $^{***}p < 0.001$.

iv) Mouse passive transfer on adult mice

This procedure is also described by Schulze et al. (2012), Hariton et al. (2017), and Hariton et al. (2023). The goal is to validate the efficacy of the AK23 IgG by quantifying the number of blistered and nonblistered hair follicle bulges in skin biopsies of mice treated with AK23 IgG versus that in the isotype control mouse IgG1.

Materials

At least six 8-week-old [second resting hair follicle (Telogen)], sex-matched C57BL/6J mice (Harlan Netherlands or equivalent)
Purified AK23 [from Basic Protocols 2 and 3; <0.4 endotoxin units (EU)/mg IgG; to check endotoxin levels, see Support Protocol 1)], 5 mg/mL
Isotype control mouse IgG1 (<0.4 EU/mg; Bio X Cell, cat. no. BP0083 or equivalent), 5 mg/mL
4% formaldehyde (Merck, cat. no. 1.00496 or equivalent)
Materials for paraffin embedding and hematoxylin and eosin staining of paraffin-embedded sections (Hariton et al., 2023)

Standard mouse facility
Light microscope (Nikon or equivalent)

1. Subcutaneously inject the back skin of the mice with 12.5 µg/g (body weight) of AK23 (at least 3 mice) or isotype control mouse IgG1 (at least 3 mice).

   Before starting any mouse experiments, approval is required from the appropriate Ethics Committee, and the experiments must conform to governmental regulations for the care and use of laboratory animals.

   To reduce stress in mice, they receive soft food (EmerAid Omnivore) placed on the cage floor after antibody injection, and they are handled using the cup-handling method. Mice must be checked regularly based on an approved score sheet, ensuring that no spontaneous hair loss or skin erosion occurs within the duration of the experiment.

2. Two days following injection, sacrifice the mice and perform tape striping (Hariton et al., 2023) to confirm weakened hair shaft anchorage in AK23-injected mice when compared to isotype control mouse IgG1–injected mice.
3. Harvest the back skin of the mice.
4. Fix the skin biopsies in 4% formaldehyde overnight and then process for paraffin embedding according to standard protocols.
5. Perform standard hematoxylin and eosin staining on 3-µm-thick skin sections (Fig. 7).
6. Count the relative number of blistered and non-blistered hair follicle bulges under a light microscope (>50 hair follicle bulges per animal; Hariton et al., 2023).

   On average, a high-quality AK23 IgG production leads to ∼70% (±10) blistered hair follicle bulges on the back skin 2 days after AK23 injection.

SUPPORT PROTOCOLS

The Support Protocols describe the following: detection of endotoxin levels in general and AK23 and isotype control mouse IgG1 antibodies (Support Protocol 1), and mycoplasma contamination and curation of the AK23 hybridoma cell stock (Support Protocol 2).

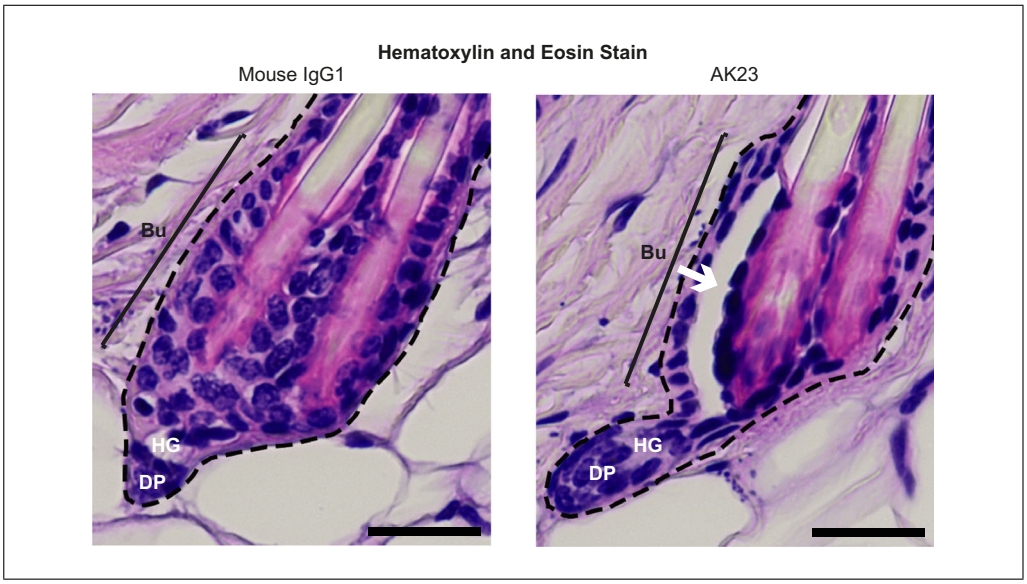

Figure 7 Hematoxylin and eosin stain of back skin of 8-week-old (second Telogen) sex-matched C57BL/6J mice injected with 12.5 µg/g (body weight) of isotype control mouse IgG1 or AK23. The white arrow indicates the blistered hair follicle bulges. Scale bars: 25 µm. Bu, Bulge; HG, hair germ; DP, dermal papilla.

SUPPORT PROTOCOL 1

DETECTION OF ENDOTOXIN LEVELS

The optimal dosage of AK23 for mice was determined to be 12.5 µg/g body weight, translating to a total requirement of 250 µg antibody injection per 8-week-old mouse weighing 20 g (Schulze et al., 2012). Given the permissible endotoxin dose of 5 EU/kg body weight, it is imperative to utilize AK23 and isotype control mouse IgG1 with endotoxin levels not exceeding 0.4 EU/mg IgG. Note that endotoxin removal with conventional kits is not efficient, possibly due to the high affinity of endotoxins for IgG; hence, it is necessary to produce the antibodies under endotoxin-free conditions.

The Limulus amebocyte lysate (LAL) test is a semiquantitative standard gel clot test for Gram-negative bacterial endotoxin, the most frequent contamination. The collection, preparation, assay, and interpretation steps of the test are described in the LAL test kit of your choice.

Materials

Solutions to be tested (especially mIgG and purified AK23)
Please note that FBS is not compatible with the LAL test; therefore, refer to the Certificate of Analysis from the FBS supplier.
Lysate multidose with sensitivity (λ) of 0.06 EU/mL (Lonza, cat. no. N283-06 or equivalent)
Control standard endotoxin kit (Lonza, cat. no. N283-06 or equivalent)
LAL reagent water (Lonza, cat. no. W50-100 or equivalent)

Borosilicate glass tubes, endotoxin-free (Lonza, cat. no. N205 or equivalent)
If necessary, tubes must be depyrogenated in an oven at 250°C for 2 h or 200°C for 4 h.
Sterile tubes with snap cap, 5 mL (Falcon polystyrene round-bottom tube 12 × 75 mm, cat. no. 352063 or equivalent)
Dry heating block set to 37°C (PeqLab or equivalent)

Perform the LAL test according to the Lonza Gel Clot LAL assay protocol to estimate the endotoxin levels in the samples being tested.

DETECTION AND REMOVAL OF MYCOPLASMA

Mycoplasma can be assessed conveniently by i) DAPI staining of plated hybridoma cells (or other cells such as keratinocytes) followed, if necessary, by ii) PCR (Fig. 8) or iii) colorimetric assays. If testing positive, iv) the cells need to be cured using commercially available kits.

NOTE: It is recommended to test hybridoma cells for mycoplasma contamination before expansion and IgG production.

Materials

AK23 hybridoma cells
Pure (100%) methanol (Merck, cat. no.179337 or equivalent)
DAPI solution (Thermo Fisher Scientific, cat. no. 62248 or equivalent)
Fluorescence mounting medium (Dako, cat. no. S302380-2 or equivalent)

T75 cell culture flasks (TPP, cat. no. 9007 or equivalent)
Microcentrifuge (Eppendorf, cat. no.5417R or equivalent)
Heating block (Eppendorf, cat. no. 5355 or equivalent)
Superfrost Plus microscope slides (Fisherbrand, cat. no. 22-037-246 or equivalent)
Square cover glasses, 18 × 18 mm (Fisherbrand, cat. no. 12-541-012 or equivalent)
Fluorescence microscope (Nikon, cat. no. Eclipse Ti-E or equivalent)

i) Testing AK23 hybridoma cells for mycoplasma by DAPI staining

1. To resuspend the cells, shake the flasks containing AK23 hybridoma cells cultured for at least 3 days for 15 s.
2. Transfer approximately 1.5 mL of supernatant with cells (1,000 cells are sufficient) into an Eppendorf safe-lock tube, and centrifuge at 400 × *g* for 7 min at room temperature.
3. Discard the supernatant.
4. Resuspend the cells in 1 mL 100% methanol containing 1 µg/mL DAPI and incubate for 15 min on a dry heating block at 37°C.
5. Centrifuge at 400 × *g* for 7 min at room temperature.
6. Discard the staining solution and add 200 µL of 100% methanol.
7. Place 50 µL of the fixed cells on a microscope slide, and let it air dry at room temperature in the dark.
8. Embed the sample with the fluorescence mounting medium, cover with a coverslip, and examine under the fluorescence microscope.

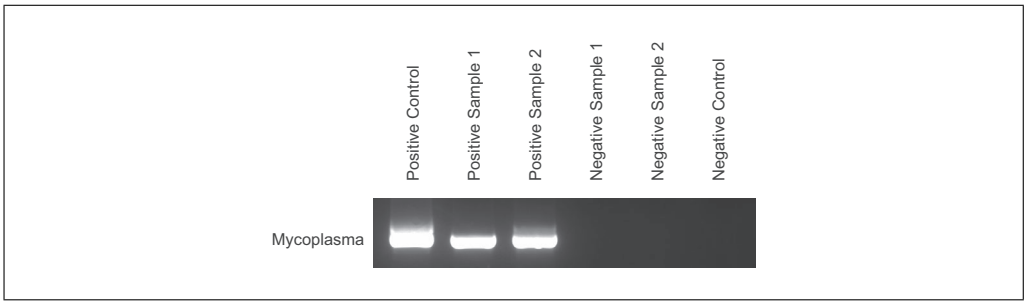

Figure 8 Agarose gel electrophoresis of PCR-amplified products using mycoplasma detection primers. A clear difference is shown between positive (contaminated) and negative (not contaminated) samples.

Note that mycoplasma contaminations are indicated by small DAPI-positive dots beside the nuclei. Uncontaminated cells exhibit nuclear fluorescence against a dark cytoplasmic background. Fluorochrome binding to mitochondrial DNA is not detectable through standard fluorescent microscopy due to low levels. In contrast, the DNA content in mycoplasmas is about 10-fold the DNA content in mitochondria and can be easily seen as bright foci against a dark background. These foci appear as pinpoints over the cytoplasm and sometimes as intercellular spaces. It is important to note that not all cells may be infected, so it is necessary to scan the preparation thoroughly before confirming that the culture is mycoplasma-free. If in doubt, proceed with PCR or colorimetric assays.

ii) Testing cells for mycoplasma contamination by PCR

PCR detection of mycoplasma is done according to a PCR mycoplasma detection kit (TaKaRa, cat. no. 6601 or equivalent).

An example of PCR mycoplasma detection is shown in Figure 8.

iii) Testing AK23 hybridoma cells for mycoplasma using colorimetric assay

A luminometric assay for mycoplasma detection is done using a mycoplasma detection kit (Lonza, cat. no. LT07-703 or equivalent).

REAGENTS AND SOLUTIONS

Preparation of PBS and PBS+

PBS and PBS+ are prepared in 0.5 M NaOH/H_2O-rinsed bottles as follows and then autoclaved.

	Chemical	Molar mass (g/mol)	Molar concentration (mM)	Weight for 1 L (g)
PBS	NaCl	58.44	136.89	8
	KCl	74.56	2.68	0.2
	KH_2PO_4	136.09	1.47	0.2
	Na_2HPO_4	141.96	4.30	0.61
PBS+	NaCl	58.44	136.89	8
(see Note)	KCl	74.56	2.68	0.2
	KH_2PO_4	136.09	1.47	0.2
	Na_2HPO_4	141.96	4.02	0.57
		Mix first (pH should be around 7.3)		
	$MgCl_2{\cdot}6H_2O$	203.30	0.98	0.2
	$CaCl_2{\cdot}2H_2O$	147.02	0.90	0.132

NOTE: To avoid salt precipitation in PBS+, it is recommended to prepare 1 bottle of solution A containing 20 × of KH_2PO_4 and Na_2HPO_4 and 1 bottle of solution B containing 20 × of NaCl, KCl, $MgCl_2{\cdot}6H_2O$, and $CaCl_2{\cdot}2H_2O$. Sterilize and store at room temperature to prevent precipitation. To dilute to 1× PBS+ add 5 mL each of solutions A and B to 90 mL of sterile distilled water. The thus-prepared 1× PBS+ can be stored at room temperature or refrigerated at 4°C.

Considering that most of the required chemicals are usually readily available at low costs, it might be significantly cheaper to prepare and test big batches of PBS and PBS+ in-house.

COMMENTARY

Background Information

PV is the generic form of the pemphigus group of autoimmune diseases, characterized by blister formation or acantholysis between basal and suprabasal layers of the oral mucosa and skin, and skin appendages such as hair follicles. The autoimmune origin of the disease was unveiled by Beutner and Jordon in 1964 (Beutner & Jordon, 1964) while the identification of the major antigenic target, Dsg3, was achieved through cDNA library screening of keratinocytes with patients' IgG 25 years later

(Amagai et al., 1991). After another 20 years, keratinocyte signaling was implicated as a novel causative factor in PV pathophysiology using plakoglobin knockout cells (Caldelari et al., 2001) coupled with PV patients' biopsies documenting keratinocyte fate conversion (Hariton et al., 2023; Williamson et al., 2006; Williamson et al., 2007). Purported in textbooks as a disease targeting the tightly packed desmosomes, PV autoantibodies primarily bind to extradesmosomal Dsg3 (Di Zenzo et al., 2016; Sato et al., 2000), reminiscent of E-cadherin or integrin-induced mechanosignaling (Anthis & Campbell, 2011; Becchetti et al., 2019). Uncoupling Dsg3 between neighboring cells through antibody binding (Heupel et al., 2009) indeed led to the identification of extradesmosomal Dsg3 as a signaling receptor (Di Zenzo et al., 2016). Currently, mechanosensing and mechanosignaling abilities of anchoring junctions, such as adherens junctions, are also discussed for desmosomal cadherins (Rubsam et al., 2017; Hariton et al., 2023; Spindler et al., 2023). However, decoding the entire signaling network downstream of uncoupled Dsg3 in PV needs in-depth systems biology–aided analyses, which can be best achieved using monospecific, pathogenic Dsg3 antibodies as a tool. Data gathered from these investigations are essential to define a causative signaling network and the hierarchy of individual pathways and contribution to acantholysis engendering the development of urgently needed first-line treatments for PV patients.

The pathogenic monospecific Dsg3 antibody AK23 represents an excellent tool for establishing a comprehensive signaling network. AK23 was produced through adoptive transfer of splenocytes from Dsg3 knockout mice immunized with recombinant mouse Dsg3 (Tsunoda et al., 2003). Of eight clonal antibodies, AK23 was the most pathogenic, recapitulating PV patients' clinical phenotype in the oral mucosa and hair follicles of mice after passive transfer (Hariton et al., 2017; Hariton et al., 2023; Schulze et al., 2012) [for PV mouse models, please refer to Hartmann and colleagues (Hartmann et al., 2023)]. Like most pathogenic PV antibodies, AK23 binds to the tip of the Dsg3 molecule in the extracellular domain 1 (EC1) involved in *cis*- and *trans*-adhesion between Dsg3 molecules of neighboring cells (Di Zenzo et al., 2012). This presumably leads to receptor-mediated signal activation through a conformational change implicating mechanotransduction (Campbell & Humphries, 2011), ultimately inducing the loosening of desmosomal adhesive strength.

AK23 has been used so far in at least 31 causative signaling studies, including ours (Rahimi et al., unpublished data), validating pathological signal activation. These studies further included the application of AK23 signaling in several PV models (Hartmann et al., 2023), such as 2D mouse and human keratinocyte cultures, 3D human skin organ cultures as well as neonatal and adult mice, replicating relevant observations in PV patients' skin (Schulze et al., 2012; Williamson et al., 2007). Where applicable, the results obtained with AK23 were also phenotypically comparable to those obtained with mucosal PV IgG (with anti-Dsg3 and without anti-Dsg1 antibodies), although the pathogenic activity is highest when polyclonal anti-Dsg3 antibodies are present (Yamamoto et al., 2007). Besides establishing a comprehensive signaling network, it will thus be of interest to not only correlate binding sites on Dsg3 with pathogenicity (Di Zenzo et al., 2012) but also affinity for Dsg3 binding with pathogenic consequences and potentially conformational changes in Dsg3.

Critical Parameters

Critical parameters for AK23 production with reproducible activity are described and explained above. Moreover, the most critical parameter for reproducible activity is the presence of bovine IgG contaminating the AK23 IgG. This caveat is avoided using FBS without bovine IgG, FBS stripped from bovine IgG, or fully synthetic media. Furthermore, it is of upmost importance to exclude endotoxin contamination for all PV models prior to starting experiments with both AK23 and isotype control mouse IgG1.

Troubleshooting

The two most common causes of low purity and activity of AK23 are as follows:

Inefficient bovine IgG stripping leads to reduced recovery of AK23 as the bovine IgG binds with higher avidity to the column than AK23 during the purification step. In this case, the stripping needs to be repeated until a ∼95% reduction in bovine IgG is achieved.

Insufficient decontamination can lead to high levels of endotoxins. In this case, it is recommended to produce a new batch of AK23 under endotoxin-free conditions. Alternatively, commercially available kits could be used for decontamination, although usually inefficient, until low levels of endotoxins are

achieved, especially if aiming to inject AK23 into mice where <0.4 EU/mg is a prerequisite for adult mice (20 g).

Understanding Results

A dose response with AK23 in a KDA on mouse or human keratinocytes is expected to show optimal fragmentation of the cellular sheet between 10 and 20 µg/mL AK23 (Fig. 6). If >50 µg/mL AK23 concentrations are necessary for maximal fragmentation, the production process is to be scrutinized (see Troubleshooting). Furthermore, it must also be considered that excess antibody results in the "hook effect" or loss of reactivity (Fig. 6), presumably due to IgG aggregation (Tate & Ward, 2004)

Time Considerations

The production of AK23 supernatant requires 7 to 8 days and the purification of AK23 via affinity purification needs 2 days. The quality control process takes ∼2 weeks.

Mouse Study Approval

Mouse experiments were approved by the ethics committee, Canton Bern, Switzerland (26/08; BE78.11; BE2_15).

Author Contributions

Eliane J. Mueller: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing—original draft; writing—review and editing. **Siavash Rahimi:** Data curation; formal analysis; investigation; methodology; resources; software; validation; visualization; writing—original draft; writing—review and editing. **Patrizia Sauta:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing—original draft; writing—review and editing. **Taravat Shojaeian:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing—original draft; writing—review and editing. **Laurence Durrer:** Methodology. **Soraya Quinche:** Methodology. **Michael Francois:** Methodology. **Elisabeth Locher:** Methodology. **Monika Edler:** Methodology; resources. **Marlies Illi:** Methodology; resources. **Thomas Gentinetta:** Methodology; resources; writing—review and editing. **Kelvin Lau:** Data curation; formal analysis; methodology; resources; visualization; writing—original draft; writing—review and editing. **Florence Pojer:** Data curation; formal analysis; methodology; resources; visualization; writing—original draft; writing—review and editing. **Luca Borradori:** Writing—review and editing. **William V. J. Hariton:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing—original draft; writing—review and editing.

Acknowledgments

The optimization of AK23 production was supported over the years by grants #31003A_135689, CRSII3_160738, and #CFII5 301202 from the Swiss National Science Foundation. We thank Prof. Masayuki Amagai and Dr. Kazuyuki Tsunoda (Keio University, Tokyo, Japan) for kindly providing the AK23-producing hybridoma cells.

Open access funding provided by Universitat Bern.

Conflict of Interest

The first author is a founder and director of the board of CELLnTEC Advanced Cell Systems AG. We used CELLnTEC media in this study. The following authors are employees of CSL Behring: Thomas Gentinetta, Marlies Illi, Monika Edler, and Elisabeth Locher. All other authors have declared no conflict of interest.

Data Availability Statement

All data reported are available in this paper. The data used to support the findings of this study or any additional information required to reanalyze the reported data will be shared upon request.

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