Supplemental Online Content

Yilmaz K, Goletz S, Pas HH, et al. Clinical and serological characterization of orf-induced immunobullous disease. *JAMA Dermatol*. Published online March 30, 2022. doi:10.1001/jamadermatol.2022.0290

This supplemental material has been provided by the authors to give readers additional information about their work.

eMethods

Indirect Immunofluorescence

For the detection of serum anti-laminin 332 reactivity a recently developed indirect IF test based on the recombinant expression of the α3, β3, and γ2 chains of laminin 332 and the recombinant laminin 332 heterotrimer on the cell surface of a human cell line (HEK293) was used as described previously (Euroimmun, Lübeck, Germany)[1-2]. In brief, all sera were subjected to the indirect IF biochip mosaic with six different substrates comprising HEK293 cells transfected with pTriEx-1 constructs for (i) LAMA3, (ii) LAMB3 (+/- His tag), (iii) LAMC2 (+/- His-tag) (encoding for the α3, β3, and γ2 chains, respectively), (iv, v) co-transfected all three plasmids encoding for the heterotrimer (+/- His tag), and (vi) the empty plasmid, as described recently[1]. All sera were applied in a 1:10 dilution in PBS supplemented with 0.2 % tween-20, and, after washing, bound autoantibodies were detected by FITC-labelled mouse IgG subclass-specific detection antibodies (IgG1, clone 8c/6-39, dilution 1:100; IgG2, clone HP-6014, dilution 1:50; IgG3, HP-6050, dilution 1:100; IgG4, clone HP-6025, dilution 1:100; all Sigma-Aldrich, St. Louis, MO, USA). Pictures were taken using a Biorevo Keyence BZ-9000 fluorescence microscope (Keyence Deutschland GmbH, Neu-Isenburg, Germany).

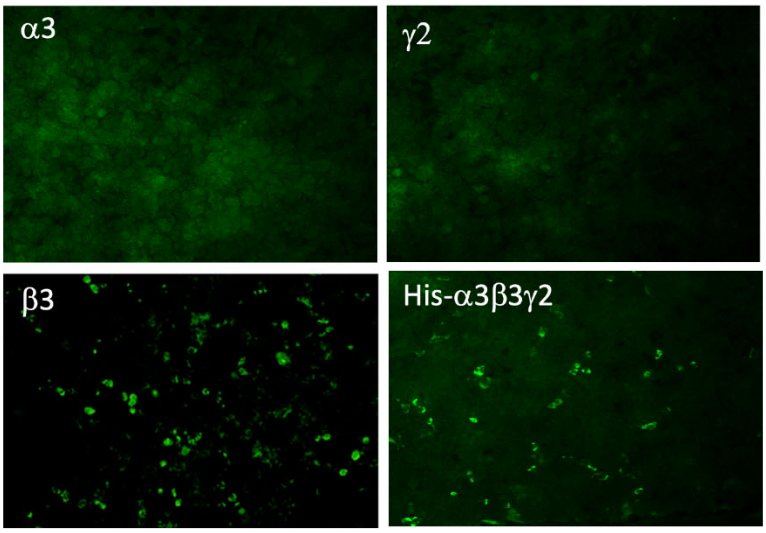
Immunoblotting

Immunoblotting was performed with (i) extract of human dermis (for reactivity against p200 protein and full-length type VII collagen), (ii) recombinant non-collagenous (NC1) domain of type VII collagen, and (iii) extracellular matrix of cultured human keratinocytes (for reactivity against laminin 332) as previously reported[3-5]. As secondary antibodies horseradish peroxidase-conjugated sheep anti-IgG1 and IgG3 antibodies were applied (Binding Site, Birmingham, UK).

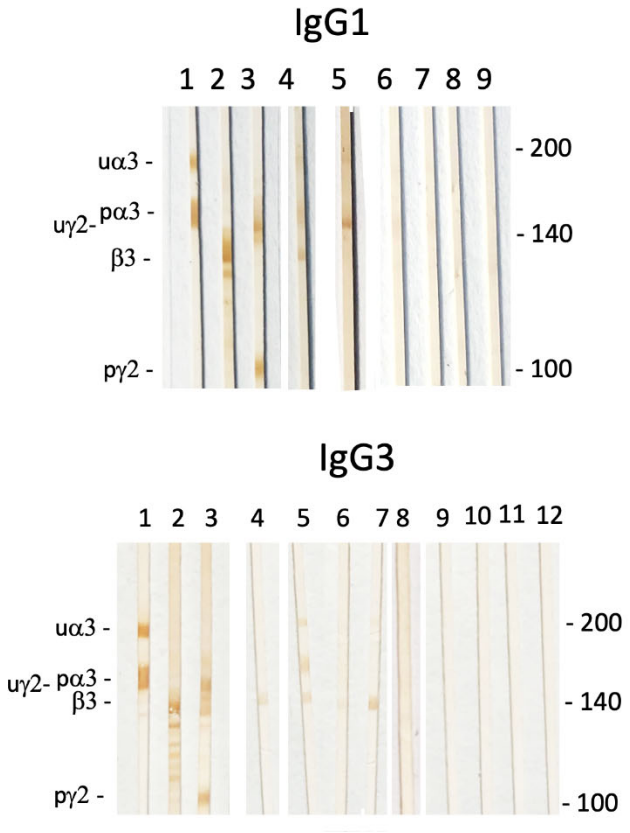
eDiscussion

The relationship between viral infections and the induction of autoimmunity has been much disputed. In this context, Blaszek *et al.* found high frequencies of Torque Teno virus (TTV) in sera of bullous pemphigoid (BP) patients as well as significant *in silico* similarity of BP180 and the virus proteins in conjunction with a cellular response to TTV-specific sequences in TTV-positive BP patients[6]. By contrast, in Case 4 (Table 1) no overlap larger than four consecutive amino acids could be detected in a protein-protein blast of four orf strains against all transcripts of laminin 332[7]. This can be attributed to a conformational epitope, as the authors correctly pointed out[7]. As previously proposed, an alternative explanation may be the alteration or unmasking of laminin 332 by orf, leading to an increased immunogenicity in predisposed individuals[7-9]. This hypothesis is in line with the explanation for solid laminin 332-expressing tumors as trigger for anti-laminin 332 MMP that is associated with malignancies in 25-30% of patients[1,10].

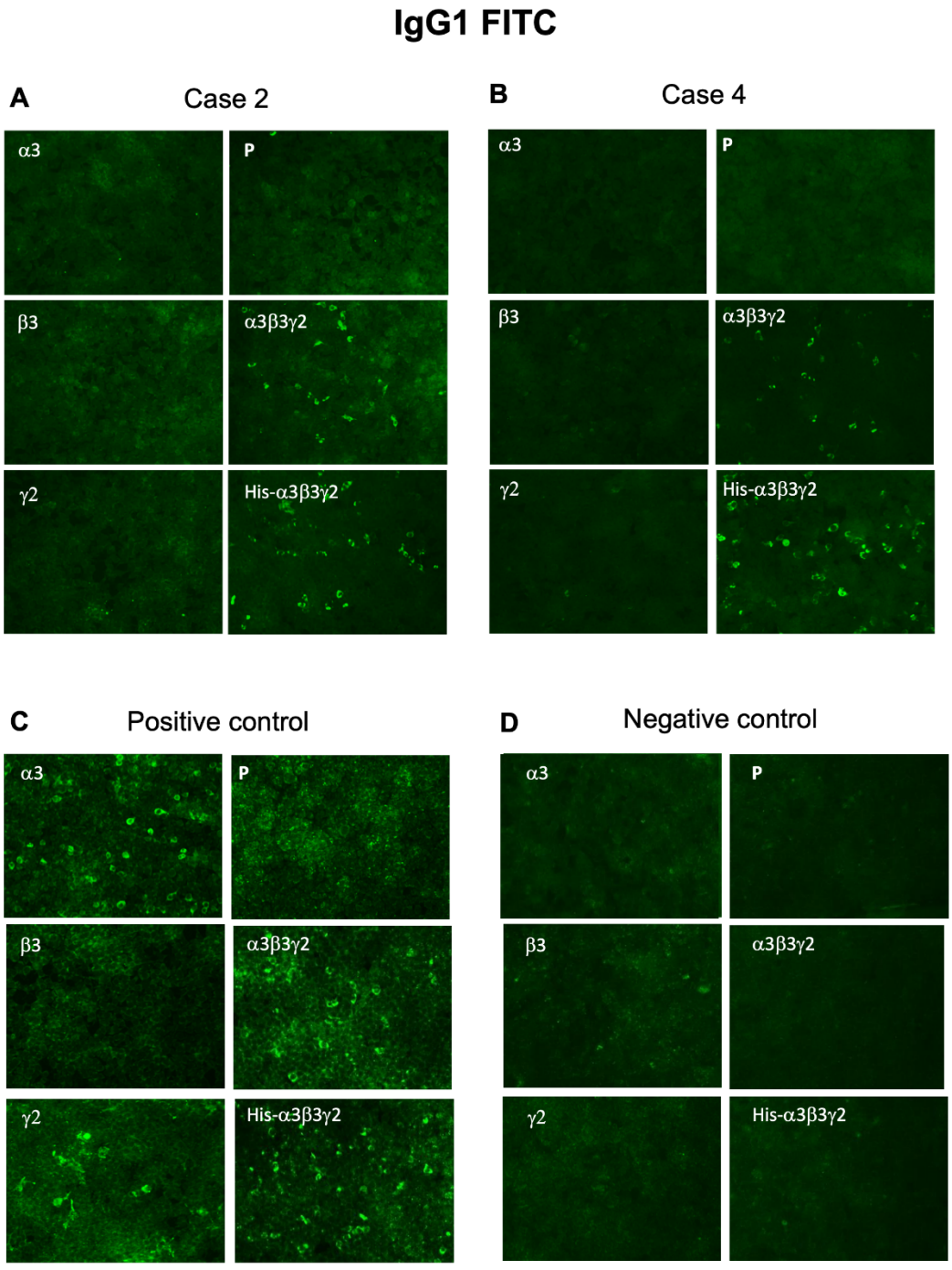
eFigure 1. Serological diagnosis of orf-induced anti-laminin 332 pemphigoid of the index patient (Case 1). Indirect immunofluorescence microscopy using membrane-bound recombinant laminin α3, β3, γ2, and the heterotrimer with His-tag expressed on HEK293 cells (His-α3β3γ2), respectively. IgG3 autoantibodies against the laminin 332 β3 chain (left lower panel) and the heterotrimer (right lower panel) are seen.



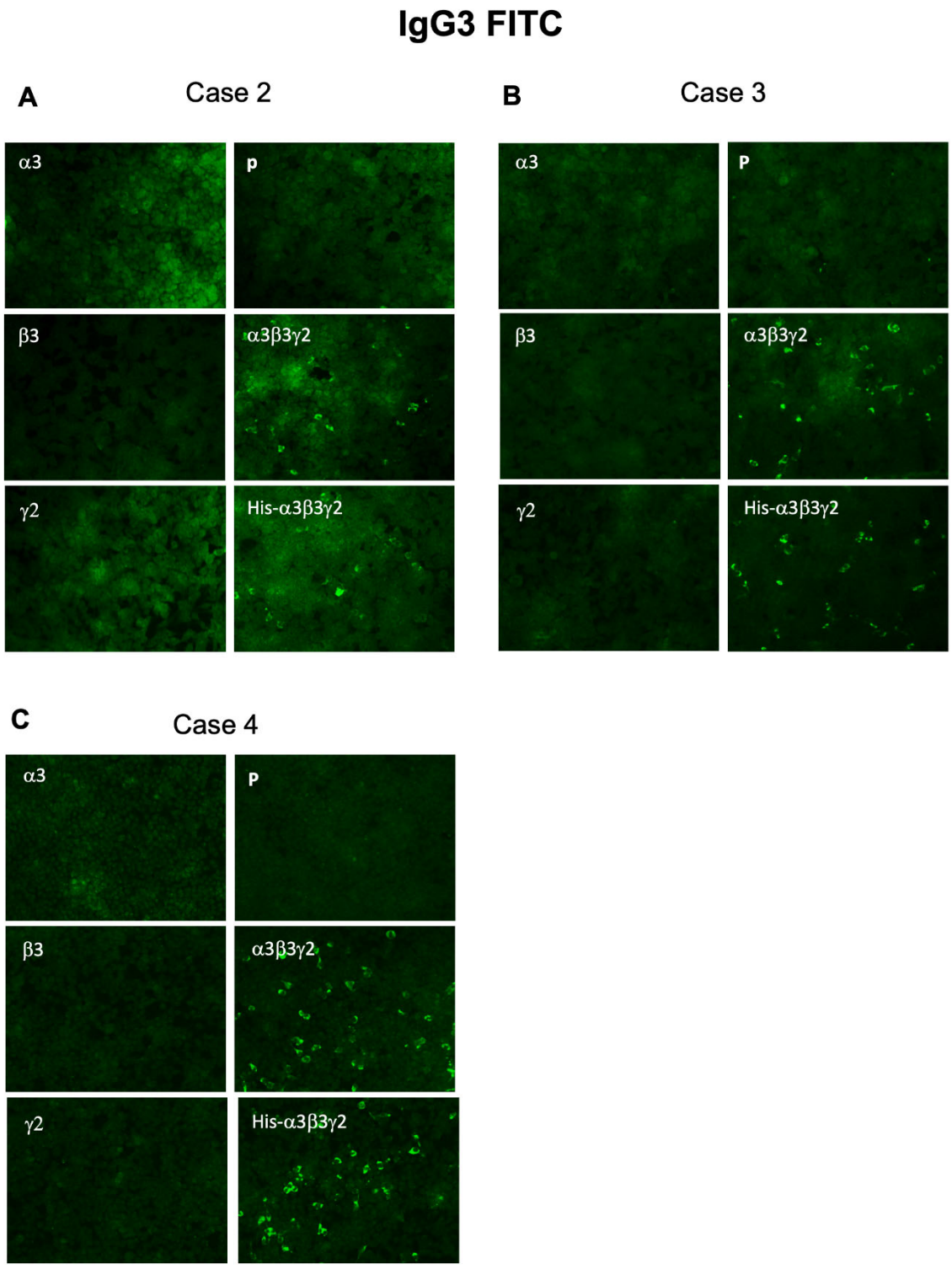
eFigure 2. Immunoblot analysis of orf-induced (bullous) pemphigoid. Representative pictures of anti-laminin 332 IgG1 (upper panel) and IgG3 reactivity (lower panel) by immunoblotting with extracts of extracellular matrix of cultured keratinocytes. **Upper panel:** Case 1 recognized the β3 and weakly the pα3 chain (lane 4) and Case 2 the uγ2 chain (lane 5). Commercial antibodies against the unprocessed (uα3) and processed α3 (pα3; lane 1), β3 (lane 2) as well as uγ2 and pγ2 chains (lane 3) served as positive controls. Sera of healthy blood donors are shown in lanes 6-9. **Lower panel:** Cases 1-4 (lanes 4-7) showed IgG3 reactivity with the β3 chain and Case 2 additional reactivity with uα3 and pα3 (lane 5). Case 5 revealed no IgG3 reactivity (lane 8). Commercial antibodies against the unprocessed (uα3) and processed α3 (pα3; lane 1), β3 (lane 2) as well as uγ2 and pγ2 chains (lane 3) served as positive controls. Sera of healthy blood donors are shown in lanes 9-12. Numbers to the right indicate molecular weight markers in kDa.



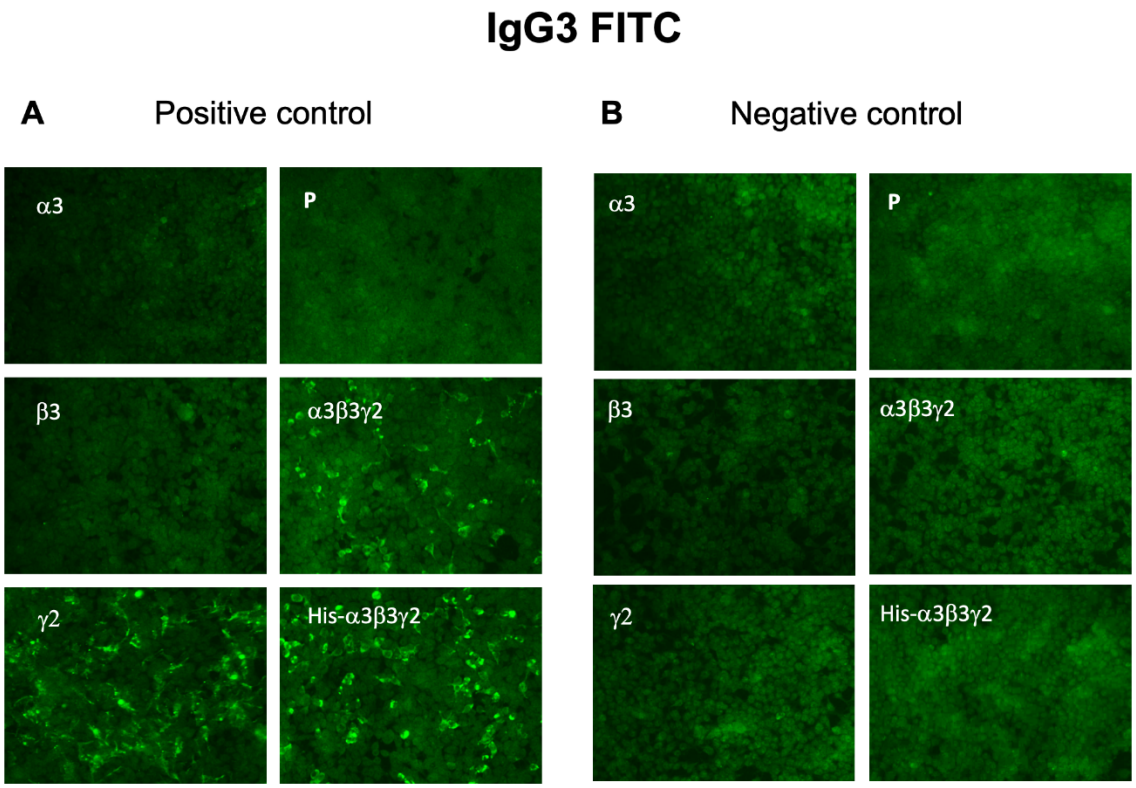
eFigure 3. IgG1 reactivity against laminin 332 by IF microscopy using Biochip™ mosaic. Detection of IgG1 autoantibodies against laminin 332 heterotrimer $\alpha3\beta3\gamma2$ (-/+ His-tag) was shown for Case 2 (**A**) and Case 4 (**B**). All other fields were negative. **C**: Serum of a patient with anti-laminin 332 mucous membrane pemphigoid (MMP) was used as a positive control. IgG1 reactivity against $\alpha3, \gamma2$ subunits as well as the heterotrimer $\alpha3\beta3\gamma2$ (-/+ His-tag) was seen. **D**: Serum of a healthy blood donor was used as a negative control. Serum dilution at 1:10 in PBS-T. Anti-IgG1 FITC antibody was diluted at 1:100 in PBS-T. P, empty plasmid.



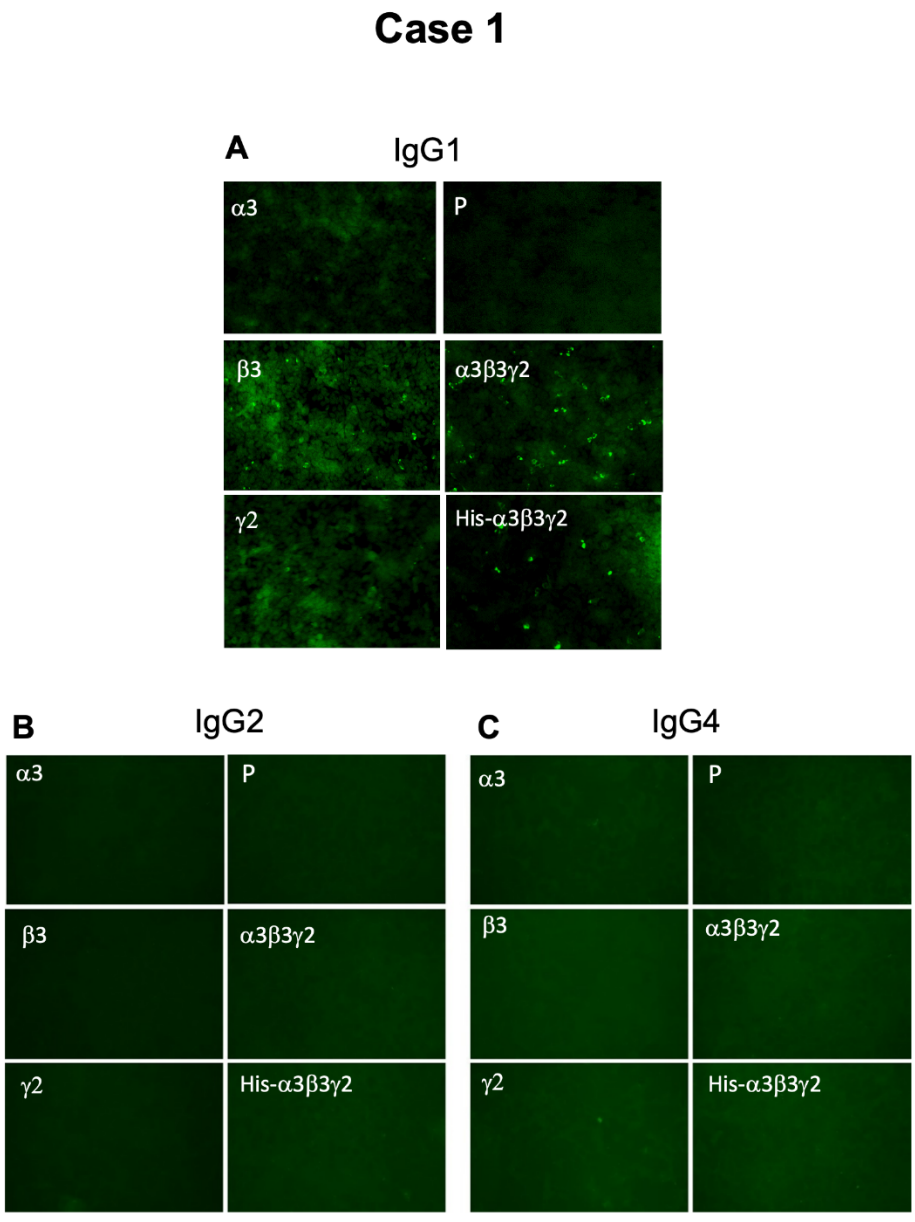
eFigure 4. Detection of IgG3 autoantibodies against laminin 332 by IF microscopy using Biochip™ mosaic. IgG3 autoantibodies against laminin 332 trimer $\alpha3\beta3\gamma2$ (-/+ His-tag) were detected in Case 2 (**A**), Case 3 (**B**) and Case 4 (**C**). In contrast, no reactivity with the single chains of laminin 332 was detected. Serum dilution at 1:10 in PBS-T. Anti-IgG3 FITC antibody was diluted at 1:100 in PBS-T. P, empty plasmid.



eFigure 5. Positive and negative controls for IgG3 reactivity against laminin 332 by IF microscopy using Biochip™ mosaic. A: Serum of a patient with anti-laminin 332 MMP was used as positive control and revealed IgG3 autoantibodies against γ2 subunit of laminin 332 and the heterotrimer α3β3γ2 (-/+ His-tag). **B**: Negative results of a healthy blood donor were shown. Serum dilution at 1:10 in PBS-T. Anti-IgG3 FITC antibody was diluted at 1:100 in PBS-T. P, empty plasmid.



eFigure 6. Analysis of IgG subclass-specific autoantibodies against laminin 332 of the index patient (Case 1) using Biochip™ mosaic. A: Serum of the index patient revealed IgG1 autoantibodies against laminin 332 heterotrimer α3β3γ2 (-/+ His-tag) and β3 chain, whereas no reactivity with α3 and γ2 chain was seen. Furthermore, no IgG2 (**B**) and IgG4 (**C**) autoantibodies could be detected. IgG3 reactivity was detailed separately in eFigure 1. Serum was diluted at 1:10 and secondary antibodies at 1:100 (IgG2, 1:50) in PBS-T. P, empty plasmid.



eTable. Clinical and immunological aspects of the 7 other hitherto published cases of orf-induced pemphigoid

Case #	Age	Sex	Interval between orf and blister formation	Direct IF BMZ	Indirect IF on Salt-split skin	ELISA/ Immunoblot	Clinical features		Reference
							Skin	Mucous membranes	
Predominant skin lesions									
6	62	f	3-4 weeks	C3	negative	not performed	++	-	[9]
7	37	m	3 weeks	C3	negative	not performed	++	-	[9]
8	n/a	n/a	2-3 weeks	C3	negative	not performed	++	-	[9]
9	n/a	n/a	2-3 weeks	C3	negative	not performed	++	-	[9]
10	n/a	n/a	2-3 weeks	C3	negative	not performed	++	-	[9]
11	51	f	16 days	IgG, C3	not performed	not performed	++	+ oral	[11]
12	42	f	4 weeks	IgG, C3	IgG1, IgG3, dermal side	Type VII collagen ELISA[a], 38 U/ml, cut-off < 20; laminin 332 Biochip™ and IB extracellular matrix, negative[b]	++	+ oral, nasal	[12]
Predominant mucosal lesions									
13	55	m	several weeks	IgG, IgM, IgA, C3 (n-serrated)	negative	all negative	+	++ oral, nasal, ocular	[13]

[a] Euroimmun, Lübeck, Germany; [b] data not shown

BMZ, basement membrane zone; IF, indirect immunofluorescence; n/a, not available

eReferences

1. Goletz S, Probst C, Komorowski L, et al. A sensitive and specific assay for the serological diagnosis of antilaminin 332 mucous membrane pemphigoid. *Br J Dermatol.* 2019;180(1):149-156.
2. Goletz S, Giurdanella F, Holtsche MM, et al. Comparison of Two Diagnostic Assays for Anti-Laminin 332 Mucous Membrane Pemphigoid. *Front Immunol.* 2021;12:773720.
3. Holtsche MM, Goletz S, von Georg A, et al. Serologic characterization of anti-p200 pemphigoid: Epitope spreading as a common phenomenon. *J Am Acad Dermatol.* 2021;84(4):1155-1157.
4. Lau I, Goletz S, Holtsche MM, Zillikens D, Fechner K, Schmidt E. Anti-p200 pemphigoid is the most common pemphigoid disease with serum antibodies against the dermal side by indirect immunofluorescence microscopy on human salt-split skin. *J Am Acad Dermatol.* 2019;81(5):1195-1197.
5. van Beek N, Kruger S, Fuhrmann T, et al. Multicenter prospective study on multivariant diagnostics of autoimmune bullous dermatoses using the BIOCHIP(TM) technology. *J Am Acad Dermatol.* 2020.
6. Blazsek A, Sillo P, Ishii N, et al. Searching for foreign antigens as possible triggering factors of autoimmunity: Torque Teno virus DNA prevalence is elevated in sera of patients with bullous pemphigoid. *Exp Dermatol.* 2008;17(5):446-454.
7. van den Bos RR, Middelburg T, van Biezen P, van der Eijk AA, Pas HH, Diercks GF. Orf-induced pemphigoid with antilaminin-332 antibodies. *Br J Dermatol.* 2012;167(4):956-958.
8. White KP, Zedek DC, White WL, et al. Orf-induced immunobullous disease: A distinct autoimmune blistering disorder. *J Am Acad Dermatol.* 2008;58(1):49-55.
9. Murphy JK, Ralfs IG. Bullous pemphigoid complicating human orf. *Br J Dermatol.* 1996;134(5):929-930.
10. Egan CA, Lazarova Z, Darling TN, Yee C, Coté T, Yancey KB. Anti-epiligrin cicatricial pemphigoid and relative risk for cancer. *Lancet.* 2001;357(9271):1850-1851.
11. Macfarlane AW. Human orf complicated by bullous pemphigoid. *Br J Dermatol.* 1997;137(4):656-657.
12. Daneshpazhooh M, Mahmoudi H, Toosi R, Tavakolpour S, Schmidt E, Zillikens D. Post-orf epidermolysis bullosa acquisita. *J Eur Acad Dermatol Venereol.* 2019;33(3):e118-e119.
13. van Lingen RG, Frank RG, Koopman RJ, Jonkman MF. Human orf complicated by mucous membrane pemphigoid. *Clin Exp Dermatol.* 2006;31(5):711-712.