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Pathogenic IgG Antibodies against Desmoglein 3 in Pemphigus Vulgaris Are Regulated by HLA-DRB1*04:02–Restricted T Cells

Rüdiger Eming,* Tina Hennerici,*† Johan Bäcklund,†,1 Claudio Feliciani,‡ Kevin C. Visconti,§ Sebastian Willenborg,* Jessica Wohde,* Rikard Holmdahl,‡ Grete Sønderstrup,§ and Michael Hertl*

Pemphigus vulgaris (PV) is considered as a model for an autoantibody-mediated organ-specific autoimmune disorder. IgG autoantibodies directed against the desmosomal cadherin desmoglein 3 (Dsg3), the major autoantigen in PV, cause loss of epidermal keratinocyte adhesion, resulting in blisters and erosions of the skin and mucous membranes. The association of human autoimmune diseases with distinct HLA alleles is a well-known phenomenon, such as the association with HLA-DRB1*04:02 in PV. However, direct evidence that HLA-DRB1*04:02–restricted autoreactive CD4+ T cells recognizing immunodominant epitopes of Dsg3 initiate the production of Dsg3-reactive IgG autoantibodies is still missing. In this study, we show in a humanized HLA-DRB1*04:02–transgenic mouse model that HLA-DRB1*04:02–restricted T cell recognition of human Dsg3 epitopes leads to the induction of pathogenic IgG Abs that induce loss of epidermal adhesion, a hallmark in the immune pathogenesis of PV. Activation of Dsg3-reactive CD4+ T cells by distinct human Dsg3 peptides that bind to HLA-DRB1*04:02 is tightly regulated by the HLA-DRB1*04:02 allele and leads, via CD40-CD40L–dependent T cell–B cell interaction, to the production of IgG Abs that recognize both N- and COOH-terminal epitopes of the human Dsg3 ectodomain. These findings demonstrate key cellular and humoral immune events in the autoimmune cascade of PV in a humanized HLA-transgenic mouse model. We show that CD4+ T cells recognizing immunodominant Dsg3 epitopes in the context of the PV-associated HLA-DRB1*04:02 induce the secretion of Dsg3-specific IgG in vivo. Finally, these results identify Dsg3-reactive CD4+ T cells as potential therapeutic targets in the future.

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PV patients with active disease, whereas healthy carriers of the PV-associated HLA class II alleles HLA-DRB1*04:02 and HLA-DQB1*05:03 are more common (4, 5).

We and others have provided evidence that these PV-associated HLA class II alleles are involved in the activation of Dsg3-specific autoaggressive CD4+ T cells, which are critical for the induction and maintenance of autoreactive memory B cells as precursors of autoantibody-producing plasma cells. Autoaggressive CD4+ Th cell responses against the ectodomain of Dsg3 were identified in PV patients by several independent investigators (6–8). Dsg3-specific autoaggressive Th2 cells were preferentially detected in PV patients with active disease, whereas healthy carriers of the PV-associated HLA class II alleles HLA-DRB1*04:02 and HLA-DQB1*05:03 showed autoreactive Th1 cell responses against Dsg3 (8). On the basis of a peptide-binding algorithm for HLA-DRB1*04:02, Wucherpfennig et al. (6) identified several Dsg3 peptides as potential T cell epitopes. We subsequently found that distinct Dsg3 peptides, which all share a positively charged arginine (R) or lysine (K) at position 4, induce a proliferative in vitro response of peripheral T cells from PV patients. Moreover, CD4+ T cell recognition of these Dsg3 epitopes was restricted by HLA-DRB1*04:02 and, in some cases, by HLA-DQB1*05:03, which share similar peptide binding motifs and thus provides a molecular basis for the dual HLA class II association observed in PV (9).

Despite the strong evidence that IgG autoAb specific for Dsg3 or Dsg1 are pathogenic in PV, the immunological mechanisms...
regulating IgG autoAb formation are largely unknown. In this study, we show in an HLA-DRB1*04:02-transgenic mouse model that induction of pathogenic, Dsg3-reactive IgG Abs requires activation of Dsg3-reactive CD4+ T cells. Moreover, T cell activation critically depends on the recognition of epitopes of the Dsg3 ectodomain, which specifically bind to the PV-associated HLA class II allele, DRB1*04:02 (6, 10). Thus T cell–dependent induction of pathogenic IgG Abs in pemphigus is tightly regulated by polymorphisms of peptide-binding motifs of distinct HLA class II alleles, which are associated with PV.

Materials and Methods

HUMANIZED HLA-DR4–TRANSGENIC MOUSE MODEL OF PEMPHIGUS

Anti-mouse CD40L mAb (MR-1) or poly hamster Ig isotype control (both BioXCell) at 500 µg i.p. on days 2, 0, 2, 4, 7, and 14 and were immunized with 20–40 µg Dsg3 i.p. on day 0.

Keratinocyte dissociation assay

The dispase based keratinocyte dissociation assay was performed as described previously (23, 24). Briefly, primary human epidermal keratinocytes were grown to confluence in CrtT-57 medium in 12-well plates (CELLLineTEC Advanced Cell Systems, Bern, Switzerland), switched to CrtT-02 medium (CELLLineTEC Advanced Cell Systems) supplemented with 1.2 mM CaCl2 24 h prior to the assay, and were incubated with mouse sera (diluted at 1:50) or the anti-Dsg3 mAb AK23 (1 µg/ml; MBL, Nagoya, Japan) overnight at 37°C. For the final 2 h of the assay, recombinant exfoliative toxin A (0.5 µg/ml; Toxin Technology, Sarasota, FL) was added. The adherent keratinocytes were then incubated with dispase I (1.5 U/ml; Roche Applied Sciences, Mannheim, Germany) at 37°C for 20 min and subjected to mechanical stress. Fragments were fixed in 1 ml of a 10% formalin solution and stained with crystal violet.

Ex vivo model using human skin biopsies

Four-millimeter punch biopsy specimens were obtained from unaffected skin samples of patients undergoing dermatosurgery in the Department of Dermatology and Allergology at the Philipps University Marburg, Germany. The patients gave written informed consent to donate excess skin samples resulting from surgical procedures for this research project. Punch biopsy specimens were incubated in 200 µl keratinocyte medium CrtT-57 (CELLLineTEC Advanced Cell Systems) supplemented with 1.2 mM CaCl2, in 96-well round-bottom plates. Serum samples of Dsg3-immunized HLA-DRB1*04:02-transgenic mice (serum samples of PBS-injected mice served as controls) were injected into the dermal site of the biopsies at a dilution of 1:20–1:25. The skin biopsies were incubated overnight at 5% CO2 and 37°C, then rinsed in PBS several times, fixed in 10% formalin, and then subjected to H&E staining for histological analysis. For detection of tissue-bound IgG Abs, cytosplasmic sections of the skin biopsies were subjected to immunofluorescence staining.

Immunofluorescence and histopathology

Human skin specimens and buccal or palatal mouse mucosa were embedded in OCT TissueTek compound (Sakura, Tokyo, Japan), frozen, cut into 3- to 4-µm sections and stained using a 1:250 dilution of a rabbit anti-mouse IgG FITC-labeled Ab for direct immunofluorescence microscopy (Zymed, San Francisco, CA). Indirect immunofluorescence with serum samples of immunized mice (1:25 dilution) was performed on monkey esophagus, according to the manufacturer’s protocol (The Binding Site, Birmingham, U.K.), and modified by using a rabbit anti-mouse IgG FITC-labeled Ab (Zymed). Formalin-fixed skin and mucosal sections of Dsg3-immunized mice were also stained with H&E.

Dsg3 ELISA

Mouse sera were diluted 1:20 and analyzed for anti-Dsg3 IgG by ELISA as described previously (20). In addition, two commercial human Dsg3 ELISA kits were also used (MBL, Nagoya, Japan; Euroimmun, Lübeck, Germany) and were modified by using an anti-mouse IgG HRP-conjugated secondary Ab.

Results

Immunization of HLA-DRB1*04:02-transgenic mice with human Dsg3 leads to the induction of Dsg3-specific, pathogenic IgG Abs

We generated a humanized mouse model aimed at reproducing the immunological key findings of PV under the immunogenetic restriction by HLA-DRB1*04:02. DBA/J1 mice transgenic for HLA-DRB1*04:02 and HLA-DQB1*03:02 (which is in a linkage disequilibrium with DRB1*04:02) and the human CD4 coreceptor, which were devoid of functional murine MHC class II (I-Ab−/−) were generated (Supplemental Fig. 1A). Mice were immunized with human recombinant Dsg3 (aa 1–566) (Table I, Supplemental Fig. 1B) and mounted a robust IgG response against human Dsg3 (Fig. 1A); these Abs belonged preferentially to the IgG1 and IgG2a subclasses (data not shown). Sera from the Dsg3-immunized mice induced loss of adhesion of monolayers of human keratinocytes (Fig. 1B) to a greater extent than sera from mice transgenic for the unrelated HLA-DRB1*04:01 allele (Supplemental Fig. 2) whereas
sera from PBS-injected HLA-DRB1*04:02–transgenic control mice did not (Fig. 1E). Injection of sera from the Dsg3-immunized HLA-DRB1*04:02–transgenic mice into human skin biopsies led to antiepithelial cell surface IgG deposits (Fig. 1C) and intraepidermal loss of adhesion, which is a hallmark of PV (Fig. 1D). In contrast, injection of sera from PBS-injected mice into human skin neither led to tissue-bound antiepithelial cell surface IgG (Fig. 1F) nor to intraepidermal loss of adhesion (Fig. 1G).

The in vivo–induced anti-human Dsg3–reactive IgG Abs recognized the same spectrum of epitopes as IgG autoantibodies from PV sera (20, 25). After the first immunization with human Dsg3, mouse sera preferentially reacted with the COOH-terminal Dsg3(EC5) subdomain and, after additional immunizations, also with the N-terminal Dsg3(EC1) and Dsg3(EC2) domains, which contain the major pathogenic B cell epitopes (Fig. 1H, I, Table I). Sera from the human Dsg3-immunized mice showed only little IgG cross-reactivity with mouse Dsg3, which shows an overall homology with human Dsg3 of 85.6% (Supplemental Fig. 3A). Despite the observed IgG reactivity of the mouse sera with several N- and COOH-terminal epitopes of the human Dsg3 ectodomain, there was no evidence for tissue-bound antiepithelial cell surface IgG Abs in oral mucosa of the Dsg3-immunized HLA-DRB1*04:02–

### Table I. Dsg3 recombinants and control proteins

<table>
<thead>
<tr>
<th>Recombinant Protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Description of Recombinant</th>
<th>Amino Acid Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dsg3 Ectodomain of human Dsg3 (EC1–5)</td>
<td>Ectodomain of human Dsg3 (EC1–5)</td>
<td>1–566</td>
<td>(8, 9, 19, 26)</td>
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<tr>
<td>Dsg3(EC1)</td>
<td>Extracellular domain 1 (EC1) of human Dsg3</td>
<td>1–161</td>
<td>(20, 52, 53, 54)</td>
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<td>Extracellular domain 2 (EC2) of human Dsg3</td>
<td>87–227</td>
<td>(20, 52, 53, 54)</td>
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<tr>
<td>Dsg3(EC3)</td>
<td>Extracellular domain 3 (EC3) of human Dsg3</td>
<td>184–349</td>
<td>(20, 52, 53, 54)</td>
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<tr>
<td>Dsg3(EC4)</td>
<td>Extracellular domain 4 (EC4) of human Dsg3</td>
<td>313–451</td>
<td>(20, 52, 53, 54)</td>
</tr>
<tr>
<td>Dsg3(EC5)</td>
<td>Extracellular domain 5 (EC5) of human Dsg3</td>
<td>424–566</td>
<td>(20, 52, 53, 54)</td>
</tr>
<tr>
<td>Dsg5</td>
<td>Ectodomain of mouse Dsg3 (EC1–5)</td>
<td>1–566</td>
<td>(55)</td>
</tr>
<tr>
<td>Col VII</td>
<td>Noncollagenous domain 1 (NC1) of human collagen VII</td>
<td>17–610</td>
<td>(56, 57)</td>
</tr>
</tbody>
</table>

<sup>a</sup>All recombinants were produced as soluble proteins in a baculovirus expression system and were purified from insect cell culture supernatants by affinity chromatography.

![FIGURE 1. Induction of human Dsg3–specific, pathogenic IgG Abs in HLA-DRB1*04:02–transgenic mice. (A) Immunization of DRB1*04:02–transgenic mice with human Dsg3 leads to a robust Ag-specific IgG response as shown by ELISA; PBS-treated mice serve as controls (n = 2). (B) In vitro, sera from Dsg3-immunized mice induce loss of adhesion of human epidermal keratinocytes as determined by dispase dissociation assay. Injection of Dsg3-immunized mouse sera into human skin specimens leads to antiepithelial surface IgG deposits (C) and intraepidermal loss of cell adhesion (D). In contrast, sera from PBS-injected mice neither induce keratinocyte monolayer dissociation (E), antiepithelial surface IgG deposits in injected human skin (F) nor loss of epidermal adhesion in these skin sections (G) ([B–G] representative results of three to five mice). (H) On day 14 after the first Dsg3 immunization, mouse sera show IgG reactivity against the COOH-terminal Dsg3(EC5) subdomain and, upon the third (day 35) immunization (I), additional IgG reactivity against the N-terminal Dsg3(EC1) and Dsg3(EC2) subdomains and to a lesser extent against Dsg3(EC3) and Dsg3(EC4) [(H and I) n = 4].](http://www.jimmunol.org/)

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transgenic mice, and accordingly, no evolving clinical phenotype (Supplemental Fig. 3B). Noteworthy, the amino acid sequences of the human and mouse Dsg3 ectodomain vary significantly in the areas of importance for CD4+ T cell activation (Table II), which may impede loss of T cell tolerance for mouse Dsg3, which precedes polyclonal activation of mouse Dsg3-specific pathogenic B cells. Comparison of the immunodominant T cell epitopes of human Dsg3 with their mouse Dsg3 analogs revealed discordant amino acids at positions that are critical MHC class II anchor motifs. For example, the mouse analog to the human immunodominant T cell epitope of Dsg3, Dsg3(97–111), which is recognized by T cells from the majority of PV patients does not share critical HLA-DRB1*04:02-binding motifs at positions 4 and 6 (Table II).

**Induction of pathogenic anti-Dsg3 IgG requires interaction of T cells and B cells**

HLA-DRB1*04:02–transgenic mice were treated with the anti-CD4 mAb GK 1.5 at the time of immunization with human Dsg3 leading to a complete inhibition of anti-Dsg3 IgG production (Fig. 2A). Furthermore, HLA-DRB1*04:02–transgenic mice were treated with the anti-CD40L mAb MR-1 before and right after immunization with human Dsg3 (Fig. 2B). Anti-Dsg3 IgG Ab production was completely inhibited, which demonstrates that T cell–B cell interaction is critical in the induction phase of Dsg3-specific IgG production. These findings in the HLA-DRB1*04:02–transgenic mouse model are in line with the clinical observation in three representative PV patients from a previously described cohort who were treated with the anti-CD20 mAb rituximab (26). Depletion of B cells by rituximab treatment (Fig. 2C) was associated with a rapid decrease of peripheral Dsg3-specific Th2 cells (Fig. 2D), followed by a more delayed decrease of anti-Dsg3 serum IgG (Fig. 2E).

**T cell recognition of desmoglein 3 peptides is tightly restricted by HLA-DRB1*04:02**

Immunization of the HLA-DRB1*04:02–transgenic mice with human Dsg3 induced a T cell response against a set of Dsg3 peptides presented by BMDC from HLA-DRB1*04:02–binding Dsg3 peptides (Table III), which all bind to HLA-DRB1*04:02 (Fig. 3B). Splenic CD4+ T cells from mice injected with human Dsg3 showed a proliferative response against a set of HLA-DRB1*04:02–binding Dsg3 peptides, respectively, but not or only to a much lesser extent with Dsg3 peptides that do not bind HLA-DRB1*04:02 (Fig. 3E, 3F). In contrast, splenic CD4+ T cells from HLA-DRB1*04:01–transgenic mice that had been immunized with the same set of HLA-DRB1*04:02–binding Dsg3 peptides (Table III) did neither show IL-4+ nor IFN-γ+ T cell responses against human Dsg3 in vitro (Fig. 3G, 3H). Immunization and in vitro restimulation with HLA-DRB1*04:02–binding Dsg3 peptides induced only an IL-4+ T cell response in a single HLA-DRB1*04:02–transgenic mouse, whereas immunization followed by in vitro challenge with HLA-DRB1*04:02–nonbinding Dsg3 peptides led to an IL-4+ and IFN-γ+ T cell response (Fig. 3G, 3H). The background stimulation of IL-4+ and IFN-γ+ lymph node T cells from HLA-DRB1*04:02–transgenic mice that were injected with PBS and adjuvant only is shown in Supplemental Fig. 4A and 4B.

**Immunization of HLA-DRB1*04:02–transgenic mice with T cell epitopes of human Dsg3 leads to the induction of anti-Dsg3 IgG**

Immunization of the HLA-DRB1*04:02–transgenic mice with a pool of HLA-DRB1*04:02–binding Dsg3 peptides (Table III) induced IgG Abs against human Dsg3 as shown by ELISA (Fig. 4A) and indirect immunofluorescence microscopy on monkey esophagus epithelium (Fig. 4B). In contrast, immunization of the HLA-DRB1*04:02–transgenic mice with Dsg3 peptides, which do not bind to HLA-DRB1*04:02 (Table III), did neither induce an IgG response against human Dsg3 by ELISA (Fig. 4A) nor did the sera react with monkey esophagus epithelium (Fig. 4C). Most strikingly, immunization of mice transgenic for HLA-DRB1*0401 (which is not related to PV and expresses different peptide binding motifs) with the HLA-DRB1*04:02–binding Dsg3 peptides did not induce IgG against Dsg3 as determined by ELISA (Fig. 4D) and indirect immunofluorescence on monkey esophagus (Fig. 4E). Neither did immunization of the HLA-DRB1*0401–transgenic mice with Dsg3 peptides that do not bind HLA-DRB1*04:02 (Table III) induce anti-Dsg3 IgG as determined by ELISA (Fig. 4D) and indirect immunofluorescence (Fig. 4F). These findings demonstrate that T cell recognition of HLA-DRB1*04:02–restricted epitopes of Dsg3 is critical for the induction of IgG Abs against Dsg3.

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**Table II. Homology of HLA-DRB1*04:02–binding peptides of the human Dsg3 ectodomain (aa 1–566) with the corresponding peptides of the mouse Dsg3 ectodomain**

<table>
<thead>
<tr>
<th>Dsg3 peptides</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>huDsg3(97–111)</td>
<td>F</td>
<td>G</td>
<td>I</td>
</tr>
<tr>
<td>mDsg3(97–111)</td>
<td>F</td>
<td>G</td>
<td>I</td>
</tr>
<tr>
<td>huDsg3(190–204)</td>
<td>L</td>
<td>N</td>
<td>S</td>
</tr>
<tr>
<td>mDsg3(190–204)</td>
<td>M</td>
<td>N</td>
<td>S</td>
</tr>
<tr>
<td>huDsg3(206–220)</td>
<td>T</td>
<td>P</td>
<td>M</td>
</tr>
<tr>
<td>mDsg3(206–220)</td>
<td>M</td>
<td>S</td>
<td>M</td>
</tr>
<tr>
<td>huDsg3(251–265)</td>
<td>C</td>
<td>E</td>
<td>C</td>
</tr>
<tr>
<td>mDsg3(251–265)</td>
<td>C</td>
<td>E</td>
<td>C</td>
</tr>
<tr>
<td>huDsg3(375–391)</td>
<td>I</td>
<td>N</td>
<td>V</td>
</tr>
<tr>
<td>mDsg3(375–391)</td>
<td>I</td>
<td>D</td>
<td>V</td>
</tr>
</tbody>
</table>

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*According to published gene sequences of human Dsg3 (3) and mouse Dsg3 (40).

*Critical amino acid anchor motifs for peptide binding to HLA-DRB1*04:02 identified by Wacherpffenig et al. (6) and Tong et al. (29) are underlined. Heterologous amino acids are bold.
of anti-Dsg3 IgG (n = 2). In addition, treatment of the HLA-DRB1*04:02–transgenic mice with the anti-CD40L Ab MR-1 at the time of immunization and immediately afterward completely inhibits anti-Dsg3 IgG Ab induction (n = 2). (C) In three PV patients, treatment with the anti-CD20 Ab rituximab does not only (C) rapidly deplete peripheral B cells but also induces (D) a rapid decrease of peripheral IL-4–secreting Dsg3-reactive Th2 cells, (E) followed by a significant reduction of anti-Dsg3 serum IgG [(C–E) referring to Ref. 26].

**Discussion**

In this study, we show in an HLA-DRB1*04:02–transgenic mouse model of the autoimmune bullous skin disorder PV that T cell recognition of epitopes of Dsg3, the autoantigen of PV, in association with HLA-DRB1*04:02, leads, via B cell help, to the formation of pathogenic IgG Abs that induce loss of epidermal keratinocyte dissociation, a key finding in PV. On the basis of these observations, PV, although pathogenetically linked to IgG autoantibodies against the desmosomal cadherin, Dsg3 should be considered as a T cell–dependent autoimmune disorder and may largely profit from a therapeutic downregulation of autoaggressive T cells.

The hypothesis that autoreactive CD4+ T cells are critical initiators and perpetuators of the autoimmune pathology of PV is based on epidemiological observations which show a strong association of PV with two distinct HLA class II alleles, DRB1*04:02 and HLA-DQB1*05:03 (4, 5, 27, 28). HLA-DRB1*04:02 possesses a negative charge at positions DR70 (aspartate) and DRβ71 (glutamate) contributing to the shape and the charge of the p4 pocket which is critical for the binding of Dsg3 peptides for presentation to autoaggressive T cells in PV (6, 10). Both, DRB1*04:02 and HLA-DQB1*05:03, show a great homology in binding epitopes of the Dsg3 ectodomain, which is supported by previous findings of our group and others that both HLA class II alleles restrict T cell recognition of identical Dsg3 epitopes (Table III) (9, 29).

Previous studies from our group identified T cell responses to Dsg3 not only in PV patients but also in healthy carriers of the aforementioned PV-associated HLA class II alleles whose activation was found to be restricted by HLA-DRB1*04:02 and HLA-DQB1*05:03, respectively (8, 19, 30). In PV, Dsg3-specific autoaggressive T cells were predominantly of the Th2 type, whereas Dsg-reactive T cells in the healthy individuals were mainly Th1 cells (7–9). A similar dichotomy of autoreactive T cells in patients and healthy donors was also found in the pathogenetically related but distinct autoimmune bullous skin disorders, pemphigus foliaceus and bullous pemphigoid, which are also mediated by pathogenic IgG autoantibodies (31, 32).

Overall, PV can be considered as a Th2-driven autoimmune disorder because most of the autoantibodies belong to the IgG4 and IgE subclasses (7, 33–37). Moreover, patients with PV have significantly lower frequencies of peripheral IL-10–secreting Dsg3-reactive type 1 regulatory T (Tr1) cells than healthy carriers of HLA-DRB1*04:02 that suppress proliferative responses of T effector cells in vitro (38). Thus, activation of Dsg3-reactive effector T cells may be controlled by Dsg3-specific Tr1 cells leading to peripheral tolerance in healthy individuals who are protected by the higher frequency of Dsg3-reactive Tr1 cells than patients (38, 39).

Immunization of humanized HLA-DRB1*04:02–transgenic mice with T cell epitopes of Dsg3 is sufficient to induce a robust CD4+ T and B cell response against human Dsg3 leading to the production of pathogenic IgG Abs, which induced loss of adhesion of human epidermal keratinocytes ex vivo and in vitro (Fig. 4A, 4B). These IgG Abs only showed little cross-reactivity with mouse Dsg3, which exhibits an overall homology to human Dsg3 of 85.6% (40). Specifically, there is a higher degree of conservation (86–89% identity) for the N-terminal EC1 and EC2 ectodomains of Dsg3, which contain the major pathogenic autoantibody epitopes in PV patients (20, 25, 40). In contrast, the homology between the human and mouse COOH-terminal Dsg3(EC3) ectodomains is much lower (56% identity) (40). Still, the in vivo–induced anti-human Dsg3-specific IgG Abs did not sufficiently bind to the relevant mouse Dsg3 epitopes to induce a clinical phenotype in our HLA-transgenic animals. Moreover, the amino acid sequences of human and mouse Dsg3 vary significantly in the areas of importance for CD4+ T cell activation (Table II), which may impede loss of T cell tolerance for mouse Dsg3, which precedes polyclonal activation of mouse Dsg3-specific pathogenic B cells. In this respect, the present mouse model differs from the human autoimmune disease PV because...
immmunization with human Dsg3 protein elicits an immune response to a foreign Ag in these animals. Thus, we are limited in analyzing loss of tolerance to endogenous mouse Dsg3 on both the CD4+ T cell and the B cell level. The current HLA-transgenic mouse model is suitable for investigating the effector phase of the autoimmune response in PV with particular emphasis on the activation and interaction of T and B cells, specific for human Dsg3, respectively. However, the primary scope of this investigation was to characterize cellular and humoral immune responses to human Dsg3 under in vivo, with emphasis on Dsg3 peptide recognition in association with HLA-B1*04:02, which is highly prevalent in PV.

FIGURE 3. CD4+ T cells from human Dsg3-immunized HLA-DRB1*04:02–transgenic mice recognize a limited set of Dsg3 peptides. (A) Splenic CD4+ T cells from Dsg3-immunized HLA-DRB1*04:02–transgenic mice recognize a set of five Dsg3 peptides that bind to HLA-DRB1*04:02 (Table III) upon coculture with HLA-DRB1*04:02+ BMDC as shown by CSFE staining. (B) In contrast, CD4+ splenic T cells from Dsg3-immunized HLA-DRB1*04:02–transgenic mice show background proliferation in coculture with a set of Dsg3 peptides that do not bind to DRB1*04:02 (Table III). CD4+ splenic T cells in coculture with BMDC alone (C) and in coculture with BMDC and the anti-CD3 Ab 145-2c11 (D) ([A–D] representative results of two experiments). (E and F) Draining lymph node cells from HLA-DRB1*04:02–transgenic mice that were immunized with the five HLA-DRB1*04:02–binding Dsg3 peptides show IL-4+ and IFN-γ+ T cell responses upon in vitro stimulation with human Dsg3 or HLA-DRB1*04:02–binding Dsg3 peptides, respectively, but not with Dsg3 peptides that do not bind HLA-DRB1*04:02. Mice immunized with HLA-DRB1*04:02–nonbinding peptides did not show an IL-4+ or IFN-γ+ T cell response to Dsg3 [(E and F) n = 6–10]. (G and H) Lymph node cells from HLA-DRB1*04:01–transgenic mice immunized with HLA-DRB1*04:02–binding and –nonbinding Dsg3 peptides, respectively, showed both IL-4+ and IFN-γ+ T cell responses to the respective set of Dsg3 peptides, but there were neither IL-4+ (except for one mouse) nor IFN-γ+ T cell responses upon in vitro restimulation with Dsg3 [(G and H) n = 2–4].
The fine specificity of the HLA-DRB1*04:02–restricted CD4+ T cell response to Dsg3 is documented by the observation that immunization of the HLA-DRB1*04:02–transgenic mice with Dsg3 peptides that do not bind HLA-DRB1*04:02 neither induces Dsg3-reactive T cell responses nor Dsg3-specific IgG Abs (Figs. 3E, 3F, 4A–C). Reversely, immunization of mice transgenic for the unrelated HLA-DRB1*04:01 allele with HLA-DRB1*04:02–binding T cell epitopes of human Dsg3 does not lead to the formation of Dsg3-specific IgG (Fig. 4D–F). HLA-DRB1*04:01, which is associated with rheumatoid arthritis, differs from DRB1*04:02 only by the positive charge of the p4 pocket, which is critical for the binding of antigenic peptides (6, 41).

Immunization of HLA-DRB1*04:01 control mice with Dsg3 protein induced Dsg3-specific IgG as measured by Dsg3 ELISA (Fig. 4D). In the functional keratinocyte dissociation assay, however, the pathogenicity of anti-Dsg3 of the HLA-DRB1*04:01 mice tended to be lower compared with serum samples of the Dsg3-immunized HLA-DRB1*04:02–transgenic mice (Supplemental Fig. 2B). Of note, we did not see principal differences in the epitope specificity of the Dsg3-reactive IgG Abs in HLA-DRB1*04:01– and DRB4:02:transgenic mice, respectively (data not shown). These findings suggest that the human Dsg3 protein also contains CD4+ T cell epitopes that bind to HLA-DRB1*04:01 and, via T cell activation and T cell–B cell interaction, induce an IgG response against the human Dsg3 Ag.

Wucherpfennig et al. (6) proposed several candidate T cell peptides of Dsg3 on the basis of an algorithm for anchor motifs of the Dsg3 peptides and the charge of critical peptide binding pockets of DRB1*04:02. Moreover, they showed a proliferative in vitro response of peripheral lymphocytes from PV patients to three of the identified HLA-DRB1*04:02–associated peptides residing within the Dsg3 ectodomain (6). An independent study confirmed these findings and identified 10 HLA-DRB1*04:02–binding epitopes of Dsg3, which included the ones proposed previously by Wucherpfennig et al. based on molecular models (28). Using long-term CD4+ T cell clones, our group demonstrated that the majority of HLA-DRB1*04:02–positive PV patients showed a proliferative T cell response to these HLA-DRB1*04:02–binding Dsg3 T cell peptides (Table III) (8, 9, 42). Specifically, all of the identified Dsg3 epitopes share common anchor residues at relative positions 1, 4, and 6, which were previously identified to be potential anchor motifs for DRB1*04:02 and carry a positive charge at position 4, which is critical for binding to the negatively charged P4 pocket of DRB1*04:02 (Table III) (6, 42).

In this study, we show that induction of anti-human Dsg3 IgG Abs in immunized HLA-DRB1*04:02–transgenic mice depends on the interaction of CD4+ T cells and B cells (Fig. 2). This in vivo finding closes an important gap that was not yet fully proven in the human disorder PV. Circumstantial evidence for a pathogenetically relevant interaction of autoreactive CD4+ T cells and B cells came from the observation that in PV patients therapeutic B cell depletion with the monoclonal anti-CD20 mAb rituximab leads to a downregulation of Dsg3-specific T cells (Fig. 2C, 2D), which is associated with a prompt clinical improvement before anti-Dsg3 serum IgG autoantibodies are decreased (Fig. 2E) (26). Noteworthy, the frequencies of tetanus toxoid (TT)–specific Th cells and serum IgG were not affected by rituximab treatment (26). This observation strongly suggests that Dsg3-reactive T cells largely depend on B cells as APCs whereas TT-specific T cells do not. This finding is in line with previous studies in rheumatoid arthritis which showed that serum IgG titers against pathogens such as pneumococcal capsular polysaccharides or TT were not significantly affected by rituximab (43). The dramatic inhibitory effect of rituximab on anti-Dsg3 IgG serum levels strongly suggests that their secretion largely depends on short-lived autoreactive plasma cells (44). Moreover, Dsg3-specific IgG-producing B cells were detected in PV patients by ELISPOT assay upon ex vivo stimulation of their peripheral lymphocytes with Dsg3 (45). When the patients’ peripheral lymphocytes were depleted of CD4+ T cells, anti-Dsg3 IgG-producing B cells were no longer detectable (45).

The critical role of T cell–B cell interaction in the PV pathogenesis is also supported by a PV mouse model established by Amagai and coworkers (46, 47). Transfer of splenocytes of Dsg3−/− mice immunized with murine Dsg3 into Dsg3+/+Rag2−/− recipient mice led to a clinical phenotype with mucosal erosions reminiscent of PV. In contrast, transfer of splenocytes depleted of CD4+ T cells to the Dsg3+/+Rag2−/− mice did neither induce autoantibody production nor a PV-like phenotype. Neither did the transfer of B cell–depleted splenocytes into Dsg3+/+Rag2−/− mice lead to the induction of anti-Dsg3 IgG and/or a clinical phenotype. Moreover, using the same animal model, Takahashi et al. (48) showed that a single Dsg3-reactive T cell clone was sufficient to prime naïve B cells to produce Dsg3-specific pathogenic IgG autoantibodies. In an independent mouse model, immunization of mice with human Dsg3 led to the induction of Dsg3-reactive Th2 cells that were able to render unprimed B cells to secrete anti-Dsg3 IgG (49).

In summary, the current study demonstrates that CD4+ T cell recognition of Dsg3, the autoantigen of PV is tightly regulated by HLA-DRB1*04:02, which is prevalent in PV. Using DRB1*04:02–transgenic mice, we show that T cell–dependent B cell activation is critical for the induction of pathogenic IgG Abs, which directly induce epidermal loss of adhesion, a key finding in the immune pathogenesis of PV. Thus, specific targeting of Dsg3-reactive CD4+ T cells holds major promise as a therapeutic option in PV. A similar approach has proven

### Table III. HLA-DRB1*04:02–binding and –nonbinding peptides of the human Dsg3 ectodomain (aa 1–566)

<table>
<thead>
<tr>
<th>Dsg3 Peptides*</th>
<th>PI1</th>
<th>PI2</th>
<th>PI6</th>
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<tbody>
<tr>
<td>Dsg3(97–111)*</td>
<td>F G  I F  V D K N T G D I N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsg3(190–204)*</td>
<td>L N S K  T A F K I V S Q E P</td>
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<td></td>
</tr>
<tr>
<td>Dsg3(206–220)*</td>
<td>T P M F  L L S R N T G E V R T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsg3(251–265)*</td>
<td>C E C N  T K V K D V N D N F P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsg3(375–391)*</td>
<td>I N V R E G  A F E P D S K T F T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsg3(85–101)**</td>
<td>Y R I S G V G I D Q P P F G I F V</td>
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<td></td>
</tr>
<tr>
<td>Dsg3(145–161)**</td>
<td>V K I L D I N D N P P V F S Q Q I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsg3(240–256)**</td>
<td>A D K D G E G L S T Q C E C N I K</td>
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<td></td>
</tr>
<tr>
<td>Dsg3(295–311)**</td>
<td>E E Y T D N W L A V Y F F T S G N</td>
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<td></td>
</tr>
<tr>
<td>Dsg3(400–416)**</td>
<td>K L V D Y I L G T Y Q A I D E D T</td>
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</tbody>
</table>

*Dsg3 15- and 17-mer peptides that bind to HLA-DRB1*04:02 (*) and Dsg3 17-mer peptides that do not bind to HLA-DRB1*04:02 (**) based on a peptide binding algorithm proposed by Wucherpfennig et al. (6) and confirmed by Tong et al. (29).

Critical amino acid anchor motifs for peptide binding to HLA-DRB1*04:02 identified by Wucherpfennig et al. (6) and Tong et al. (29) are underlined.
also thank Dr. Roland Martin for thoughtful discussion and critical comments on the manuscript.

Disclosures

R.E. has consultant arrangements with and has received payments for lectures from Novartis and received grants and payments for lectures and travel expenses from Fresenius Medical Care; M.H. has board memberships with Novartis and GlaxoSmithKline Stiefel; has consultant arrangements with Roche, Biogen Idec, and Union Chimique Belge, received payments for lectures from Janssen-Cilag and Biogen Idec, and received travel support from Janssen-Cilag; and J.W. is now employed by Millenyi Biotec. The other authors have no financial conflicts of interest.

References


Supplemental Figure 1. HLA-DRB1*04:02-transgenic mice and immunization protocols to study T cell and IgG responses against human desmoglein 3 (Dsg3). (A) Expression of transgenes shown as histograms: HLA-DRA1*01:01-DRB1*04:02 /-DQA1*03:01-DQB1*03:02 (DQ8)-transgenic mice on a DBA/1J background express the human CD4 co-receptor (L120), HLA-DR (L243), HLA-DQ (Tu169) and are deficient of mouse MHC class II (IA-ß, 25-9-17) (solid line) in contrast to wildtype mice that do neither express human CD4, HLA-DR nor HLA-DQ but express mouse MHC class II (IA-ß) (dotted line). (B) For the induction of Dsg3-reactive IgG, mice were immunized by intraperitoneal injection of recombinant human Dsg3 (20-40 µg) in alum on day 0 followed by immunizations with 20-40 µg Dsg3 on days 14 and 28. Sera were collected on days 0, 14, 21, 28, 35 and 49. (C) For the induction of Dsg3-reactive T cell responses, mice were injected subcutaneously into the hind paws with human Dsg3 in adjuvant (20-40 µg, incomplete Freund’s adjuvans (IFA) or TiterMax on days 0 and draining popliteal and inguinal lymph nodes were harvested on day 7-10.

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Supplemental Figure 2. Sera from HLA-DRB1*04:02-transgenic mice immunized with human desmoglein 3 (Dsg3) induce dissociation of human epidermal keratinocyte monolayers to a greater extent than sera from Dsg3-immunized HLA-DRB1*04:01-transgenic mice. (A) HLA-DRB1*04:02- and (B) HLA-DRB1*04:01-transgenic mice, respectively, were immunized with human Dsg3 protein (Suppl. Fig. 1C). Sera from these mice prior to Dsg3 immunization (day -1), after one (day14) and three (day 49) Dsg3-immunizations, respectively, were added to monolayers of cultured human epidermal keratinocyte in a dispase dissociation assay. Shown are numbers of keratinocyte fragments (n= 5 mice/group).
Supplemental Figure 3. IgG cross-reactivity against mouse desmoglein 3 (Dsg3) of sera from HLA-DR04:02-transgenic mice that were immunized with human Dsg3. Sera from HLA-DR04:02-transgenic animals that had been immunized with human Dsg3 protein were assayed for IgG reactivity against mouse and human Dsg3 (Table 1). (A) By immunoblot analysis, sera show IgG against human Dsg3, and to a much lesser extent, against mouse Dsg3. Human collagen VII (Col VII) served as an irrelevant control protein. (B) Accordingly, direct immunofluorescence microscopy of oral mucosa (tongue) from HLA-DR04:02-transgenic mice immunized with human Dsg3 does no show tissue-bound anti-epithelial cell surface IgG (representative results out of 5 mice).
Supplemental Figure 4. Proliferative response to human Dsg3 and Dsg3 peptides of draining lymph node T cells from PBS-injected HLA-DRB1*04:02-transgenic mice. (A, B) Popliteal and inguinal lymph node T cells from HLA-DRB1*04:02-transgenic mice that were injected with PBS/IFA neither show IL-4+ (A) nor IFNγ+ (B) responses in vitro against human Dsg3, the HLA-DRB1*04:02-binding or HLA-DRB1*04:02 non-binding Dsg3 peptides, respectively (Table 2). Controls include in vitro challenge with human collagen VII (Col VII) and PHA (Table 1), respectively (n= 2 mice)