Dichotomy of Autoreactive Th1 and Th2 Cell Responses to Desmoglein 3 in Patients with Pemphigus Vulgaris (PV) and Healthy Carriers of PV-Associated HLA Class II Alleles

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Dichotomy of Autoreactive Th1 and Th2 Cell Responses to Desmoglein 3 in Patients with Pemphigus Vulgaris (PV) and Healthy Carriers of PV-Associated HLA Class II Alleles

Christian Veldman,* Angelika Stauber,* Ralf Wassmuth, † Wolfgang Uter, ‡ Gerold Schuler,* and Michael Hertl**

Pemphigus vulgaris (PV) is the most severe autoimmune bullous skin disorder and is primarily associated with circulating autoantibodies (autoAb) against desmoglein 3 (Dsg3). In light of recent evidence that autoreactive T cells are critical for the induction and regulation of Ab production, the goal of this study was to characterize and quantitate autoreactive T cells in patients with PV and healthy controls. Peripheral Dsg3-reactive Th cells from 28 patients with acute-onset, chronic active, and remittent PV were quantitated by MACS secretion assay. Dsg3-reactive Th2 cells were detected at similar frequencies in all studied PV patients, while the number of autoreactive Th1 cells exceeded those of Th2 cells in chronic active PV. In contrast, healthy carriers of the PV-associated HLA class II alleles, DRB1*0402 and DQB1*0503, exhibited exclusively Dsg3-reactive Th1 cell responses, while healthy carriers of other HLA class II alleles did not. Moreover, the presence of IgG1 and IgG4 against Dsg3 was directly related to the ratio of Dsg3-reactive Th1/Th2 cells. T cell recognition of Dsg3 was restricted by HLA-DRB1*0402 and DQB1*0503 in PV patients and Dsg3-responsive healthy donors. These observations strongly suggest 1) that the appearance of Dsg3-reactive Th2 cells is restricted to patients with PV; 2) that specific HLA class II alleles that are prevalent in PV are critical for T cell recognition of Dsg3 in PV patients and Dsg3-responsive healthy donors; and 3) that autoAb production is associated with both Th1 and Th2 cells. The Journal of Immunology, 2003, 170: 635–642.

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To better understand the pathogenic role that T cells play in the immune pathogenesis of PV, we assessed in this study whether distinct autoreactive Th cell subsets were associated with the clinical activity of PV and whether T cell recognition of Dsg3 was restricted by specific (i.e., PV-associated) HLA class II alleles. Peripheral Dsg3-reactive Th1 and Th2 cells from 28 PV patients and 25 healthy controls were isolated and quantitated by MACS secretion assay. Furthermore, HLA class II restriction of Dsg3-autoreactive Th cells from patients and healthy donors was thoroughly characterized. The findings of this study suggest that Dsg3-reactive Th2 cells are restricted to patients with PV, and that distinct (i.e., PV-associated) HLA class II alleles are critical for T cell recognition of Dsg3 in PV patients and healthy donors.

**Materials and Methods**

**Patients and controls**

Heparinized blood samples (40–80 ml) were obtained from a total of 28 adult patients with acute-onset (n = 9), chronic active (n = 8), and remit-tent PV (n = 11) who were seen at the Dermatology Department of University of Erlangen as well as from 25 healthy control individuals. The clinical diagnosis of PV was confirmed by 1) histopathology (suprabasal acantholytic blisters), 2) direct immunofluorescence microscopy (epidermal intercellular IgG and/or C3 deposits in perilesional skin), and 3) detection of circulating autoAb by indirect immunofluorescence microscopy (intercellular IgG binding to epithelial cells of monkey esophagus) and/or the presence of Borrelia burgdorferi antibody against Dsg3 that were detected by a commercial ELISA (MDL, Naka-ku Nagoya, Japan; Table I). PV patients with cutaneous blisters had also autoAb against Dsg1 (Table I). Acute-onset PV was defined as the de novo development of blisters/erosions on previously unaffected mucosal surfaces and/or skin; all the studied patients with acute PV had not yet received immunosuppressive therapy. Chronic active PV was described as the expansion/persistence of existing blisters or erosions on mucosal surfaces and/or skin; some of these patients had already received immunosuppressive treatment. Patients with remittent PV had not experienced new mucosal blisters/erosions for ≥ 6 mo before the study (Table I). HLA class II genotyping was performed in all patients and controls. The determination of HLA-DRB1 and DQB1 alleles was conducted at high resolution by enzyme-linked probe hybridization assay (Biotest, Dreieich, Germany) using locus-specific PCR products as templates. All PV patients and healthy control donors gave written consent to participate in this study.

**Production and purification of human recombinant Dsg3**

The recombinant protein PVHis, a fusion protein consisting of the EC1–5 domain of Dsg3 linked to an E tag and a histidine tag, was used as a source of antigenic material for the production of PVHis in SF21 insect cells as described previously (13). For the production of Dsg3 protein, 3 × 10⁷ High-Five insect cells were inoculated with PVHis baculovirus at a multiplicity of infection of 10. Culture supernatants of baculovirus-infected insect cells were collected after 4 days, and Dsg3 protein was purified from culture supernatants over nickel-nitrilotriacetic acid-linked agarose (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

**Quantitation of Dsg3-reactive Th1 and Th2 cells by MACS cytokine secretion assay**

Autoreactive T cells were isolated by MACS following the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, autoreactive Th1 cells (secretting IFN-γ) and Th2 cells (secretting IL-4) were isolated from short term (16–h) cultures with 6–8 × 10⁵ PBMC of PV patients/controls and 10 μg/ml Dsg3. Upon termination of the cultures, high affinity anti-cytokine Ab were added that bound to the surface of the cytokine-secreting T cells, and labeling with a secondary magnetic bead-coupled Ab allowed for specific enrichment of IL-4- or IFN-γ-secreting T cells by passage over magnetic columns (21) that were finally counted in a hemocytometer. IFN-γ-secreting Th cells were classified as Th1 cells and IL-4-secreting Th cells as Th2 cells.

**Propagation of Th1 and Th2 cells and proliferative T cell assays**

Following isolation by MACS assay, Dsg3-reactive Th1 and Th2 cells were cloned by limiting dilution and were expanded by repeated stimulation with 1% PHA (Life Technologies) and x-irradiated (50 Gy) PBMC as APC as described previously (13, 22). For proliferative assays, human T cells were cultured in a medium consisting of RPMI 1640 (Life Technologies) with 10% heat-inactivated pooled human serum (Life Technologies), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 20 mM l-glutamine. Th cells (5 × 10⁵) were cultured in duplicate with 5 × 10⁴ x-irradiated (50 Gy) autologous PBMC in 200-μl round-bottom microtiter plates (Costar, Cambridge, MA) for 72 h at 37°C in 5% CO₂. T cell proliferation was determined by the incorporation of [³H]thymidine (DuPont, Mechelen, Belgium) that was added for the last 18 h of the culture. T cell proliferation was expressed as a stimulation index (SI), which is the ratio of [³H]thymidine uptake (cpm) in cultures with Ag to that in cultures without Ag. SI values >3 were considered significant. In addition, 100 μl of supernatant were removed from each cultured for the analysis of immunoreactivity against IL-4 and IFN-γ according to the manufacturer’s instructions (BD PharMingen, Heidelberg, Germany).

**HLA restriction assays**

Cloned T cells (5 × 10⁴) were cocultured for 72 h with 5 × 10⁴ x-irradiated (80 Gy) autologous or HLA-matched B lymphoblastoid cell line (B-LCL) cells and 10 μg/ml of Dsg3 for 72 h, and T cell proliferation was determined by the incorporation of [³H]thymidine (DuPont) that was added for the final 18 h of the culture (23). For the HLA blocking experiments, mAb against HLA-DR (clone L243), HLA-DQ (clone SK10), or HLA-DP (clone B7/21; at 50 ng/well; all from BD PharMingen) were added to the cocultures with Dsg3-reactive T cell clones and B-LCL as APC. Again, the proliferative T cell response to Dsg3 was determined by the incorporation of [³H]thymidine (DuPont) that was added for the final 18 h of the culture.

**Statistical analyses**

In addition to descriptive uni- and bivariate statistics, the association between the clinical activity of PV and Dsg3-reactive Th response and the autoAb profile, respectively, was statistically tested with the Kruskal-Wallis test (Fig. 2). In view of sparse and skewed data, which render asymptotic test results unreliable, exact p values were computed. Correlation was analyzed using the Spearman rank correlation coefficient. The statistical software package SAS (version 8.2, SAS Institute, Cary, NC) was used for data analysis.

**Results**

**Frequency analysis of Dsg3-reactive Th1 and Th2 cells in PV patients**

The majority (23 of 28) of the studied PV patients expressed either HLA-DRB1*0402 (39%) or HLA-DQB1*0503 (43%), HLA class II alleles that were prevalent in PV (Table I). Independent from the clinical activity and the immunosuppressive treatment of PV, all PV patients mounted detectable Th2 responses against Dsg3, ranging from 3.2–9.6 cells/10⁵ PBMC (Fig. 1A). Statistical analysis revealed that there were no significant differences in the Th2 frequency in acute-onset, chronic active, or remittent PV (Fig. 2A). In contrast, Dsg3-reactive Th1 cells responses that were also identified in all the PV patients varied in frequency in the studied subgroups (Fig. 1A). The eight patients with chronic active PV mounted Dsg3-reactive Th1 cell responses that were significantly higher than those in the groups of PV patients with acute-onset and remittent disease (Fig. 2A). Since variation in Th1 frequency among the three disease classes was significant (p = 0.016) and variation in Th2 frequency was not (p = 0.36; Fig. 2A) we sought to assess whether the Th1/Th2 ratio related to the levels of Dsg3-reactive IgG. In fact, there was a strong correlation between the Th1/Th2 ratio and IgG1 (0.82), IgG4 (0.66), as well as total IgG autoAb (0.73) reactive with Dsg3, which was significant (p < 0.0001) for all these parameters (Fig. 2B). Noteworthy, there was no correlation between the ratio of Dsg3-responsive Th1/Th2 cells and IgG titers against Dsg1 (0.05; p = 0.82) that were present in some of the patients’ sera. There was also no relationship between the frequency of Dsg3-reactive Th1 cells and Dsg3-reactive IgG in the healthy donors, since Dsg3-reactive autoAb were not detected (not shown).
<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>HLA Class II Alleles</th>
<th>Clinical Phenotype</th>
<th>Medication (per day)</th>
<th>Auto-Ab Profile (IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV1 Acute onset</td>
<td>DRB1: 0401, 1401, DQB1: 0301, 0503</td>
<td>Erosions of the oral mucosa</td>
<td>None</td>
<td>3.1</td>
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<td>PV2 Acute onset</td>
<td>DRB1: 1301, 1404, DQB1: 0603, 0503</td>
<td>Erosions of the palate</td>
<td>None</td>
<td>42.4</td>
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<td>PV3 Acute onset</td>
<td>DRB1: 0401, 1303, DQB1: 0301, -</td>
<td>None</td>
<td>None</td>
<td>7.5</td>
</tr>
<tr>
<td>PV4 Acute onset</td>
<td>DRB1: 0801, 1301, DQB1: 0402, 0603</td>
<td>Erosions of the oral mucosa</td>
<td>None</td>
<td>1.4</td>
</tr>
<tr>
<td>PV5 Acute onset</td>
<td>DRB1: 0701, - , DQB1: 02, -</td>
<td>None</td>
<td>None</td>
<td>28.3</td>
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<tr>
<td>PV6 Acute onset</td>
<td>DRB1: 0402, 04, DQB1: 0302, 0304</td>
<td>Multiple herpetiform and tense cutaneous blisters</td>
<td>None</td>
<td>20.8</td>
</tr>
<tr>
<td>PV7 Acute onset</td>
<td>DRB1: 0402, 00, DQB1: 0302, 0301</td>
<td>Flaccid blisters on trunk</td>
<td>None</td>
<td>75</td>
</tr>
<tr>
<td>PV8 Acute onset</td>
<td>DRB1: 0803, 1405, DQB1: 0503, 0601</td>
<td>Extensive blisters and erosions of the entire integument</td>
<td>None</td>
<td>129</td>
</tr>
<tr>
<td>PV9 Acute onset</td>
<td>DRB1: 0402, 0405, DQB1: 02, 0302</td>
<td>Erosions of the scalp</td>
<td>None</td>
<td>27.8</td>
</tr>
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<td>PV10 Acute onset</td>
<td>DRB1: 0402, 0401, DQB1: 0302, 0301</td>
<td>None</td>
<td>None</td>
<td>6.1</td>
</tr>
<tr>
<td>PV11 Acute onset</td>
<td>DRB1: 0801, 1301, DQB1: 0402, 0603</td>
<td>None</td>
<td>Chronic erosions of the buccal mucosa</td>
<td>100 mg AZA, 100 mg DADPS, 10 mg MP</td>
</tr>
<tr>
<td>PV12 Acute onset</td>
<td>DRB1: 0803, 1401, DQB1: 0402, 0603</td>
<td>None</td>
<td>None</td>
<td>52 mg MP, 100 mg AZA</td>
</tr>
<tr>
<td>PV13 Acute onset</td>
<td>DRB1: 0804, 1411, DQB1: 0402, 0503</td>
<td>Chronic erosions on the face</td>
<td>None</td>
<td>76.4</td>
</tr>
<tr>
<td>PV14 Acute onset</td>
<td>DRB1: 0803, 1401, DQB1: 02, 0503</td>
<td>None</td>
<td>None</td>
<td>80 mg MP.</td>
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<td>PV15 Acute onset</td>
<td>DRB1: 0402, 0405, DQB1: 02, 0302</td>
<td>None</td>
<td>Erosions of the tongue</td>
<td>100 mg AZA, 50 mg DADPS, 25 mg MP</td>
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<tr>
<td>PV16 Acute onset</td>
<td>DRB1: 0402, 1104, DQB1: 0302, 0301</td>
<td>None</td>
<td>Chronic buccal erosions and ulcers</td>
<td>100 mg AZA, 50 mg DADPS, 10 mg MP</td>
</tr>
<tr>
<td>PV17 Acute onset</td>
<td>DRB1: 0803, 1405, DQB1: 0503, 0601</td>
<td>Extensive crusted erosions of the trunk</td>
<td>None</td>
<td>50.6</td>
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<tr>
<td>PV18 Remittent</td>
<td>DRB1: 0301, 0402, DQB1: 02, 0302</td>
<td>None (vegetating erosions of the inguinal folds)</td>
<td>None</td>
<td>76.8</td>
</tr>
<tr>
<td>PV19 Remittent</td>
<td>DRB1: 0402, 1104, DQB1: 0302, 0301</td>
<td>None</td>
<td>None (buccal erosions/ulcers)</td>
<td>150 mg AZA, 50 mg DADPS, 25 mg MP</td>
</tr>
<tr>
<td>PV20 Remittent</td>
<td>DRB1: 0301, 0402, DQB1: 02, 0302</td>
<td>None (initially flaccid blisters on the trunk)</td>
<td>None (initially extensive oral erosions)</td>
<td>100 mg AZA</td>
</tr>
<tr>
<td>PV21 Remittent</td>
<td>DRB1: 1401, 1502, DQB1: 0503, 0601</td>
<td>None</td>
<td>None (erosions of the hard palate)</td>
<td>4 mg MP, 100 mg AZA</td>
</tr>
<tr>
<td>PV22 Remittent</td>
<td>DRB1: 0401, 0804, DQB1: 0302, 0402</td>
<td>None</td>
<td>None (oral erosions/ulcers)</td>
<td>None</td>
</tr>
<tr>
<td>PV23 Remittent</td>
<td>DRB1: 1401, 0405, DQB1: 0503, 0302</td>
<td>None</td>
<td>None (buccal erosions/ulcers)</td>
<td>10 mg LF, 7.5 mg MP</td>
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<tr>
<td>PV24 Remittent</td>
<td>DRB1: 0804, 1411, DQB1: 0402, 0503</td>
<td>None (erosions on the face, trunk, and extremities)</td>
<td>None (oral and labial erosions)</td>
<td>25 mg PD, 100 mg AZA</td>
</tr>
<tr>
<td>PV25 Remittent</td>
<td>DRB1: 0402, 1104, DQB1: 0302, 0301</td>
<td>None</td>
<td>None (buccal and gingival erosions/ulcers)</td>
<td>7.5 mg PD</td>
</tr>
<tr>
<td>PV26 Remittent</td>
<td>DRB1: 0803, 1405, DQB1: 0503, 0601</td>
<td>None</td>
<td>None (oral erosions)</td>
<td>None</td>
</tr>
<tr>
<td>PV27 Remittent</td>
<td>DRB1: 0101, 1401, DQB1: 0501, 0503</td>
<td>None</td>
<td>None (oral erosions)</td>
<td>8 mg MP, 100 mg AZA</td>
</tr>
<tr>
<td>PV28 Remittent</td>
<td>DRB1: 0402, 1301, DQB1: 0302, 0603</td>
<td>None (erosions of the scalp)</td>
<td>None (discrete buccal erosions)</td>
<td>None</td>
</tr>
</tbody>
</table>

* As classified in Materials and Methods.
* a As determined by ELISA with recombinant Dsg1 and Dsg3, respectively. Values are expressed as the index value ([A_{sample} - A_{negative}] / [A_{positive} - A_{negative}] × 100). Values >14 (anti-Dsg1) and >46 (anti-Dsg3) were considered significant.
* b The clinical description of mucosal or cutaneous lesions in parentheses refers to the initial clinical phenotype.
Frequency analysis of Dsg3-reactive Th cells in healthy individuals

Thirteen of 28 randomly chosen healthy donors carried the PV-prevalent HLA class II alleles, HLA-DRB1*0402 and/or HLA-DQB1*0503, while the remaining 15 donors expressed PV-unrelated HLA class II alleles (Fig. 1B). None of the healthy donors had circulating autoAb against Dsg3 as determined by ELISA or immunoblot with recombinant Dsg3, as described recently (24). All healthy carriers of the PV-associated HLA class II alleles, HLA-DRB1*0402 and HLA-DQB1*0503 (Co18 carried both HLA alleles), exhibited T cell reactivity against Dsg3 that was exclusively of the Th1 type (Fig. 1B). In these healthy donors, Dsg3-reactive Th1 cells were detected at frequencies similar to those of the PV patients (Fig. 1B). In contrast, none of the healthy carriers of PV-unrelated HLA class II alleles exhibited Dsg3-specific Th1 or Th2 responses (Fig. 1B).

Cytokine profile of long term cultured Dsg3-reactive Th1 and Th2 cell clones isolated by MACS secretion assay

The Ag specificity and cytokine profile of Dsg3-reactive Th1 and Th2 cell clones that were isolated by MACS secretion assay was investigated upon in vitro culture of T cells. MACS-selected Th1 cell clones (n = 13) from three PV patients continued to secrete IFN-γ, but no IL-4, upon stimulation with Dsg3 (Fig. 3A), while autoreactive Th2 cell clones (n = 18) from three PV patients secreted IL-4, but no IFN-γ, upon in vitro challenge with Dsg3. Shown in Fig. 3A are representative data for five Th1 and four Th2 clones from the PV patient PV1. Accordingly, a total of 16 Dsg3-reactive Th1 clones from three Dsg3-reactive healthy donors maintained their cytokine profile (i.e., secretion of IFN-γ, but not IL-4) upon in vitro challenge with Dsg3; shown in Fig. 3B are representative data for nine Th1 clones from the healthy donor Co16. Upon long term culture (i.e., more than third Ag stimulation), most autoreactive T cell clones from PV patients and healthy controls showed a decreased proliferative in vitro response to Dsg3 (Fig. 3, A and B). Noteworthy, all the Th clones retained their cytokine profile, i.e., Th1 clones produced exclusively IFN-γ and Th2 clones secreted IL-4, but no IFN-γ. There may have been a potential bias toward the selection of Th1 cells that produced high amounts of IFN-γ, but not of Th1 cells that produced low amounts of IFN-γ. We have not performed titration studies to evaluate the efficacy of the MACS secretion assay to detect Th1 cells that produce little IFN-γ. However, selective isolation of T cells that produced high IFN-γ seems rather unlikely, since the MACS-selected Th1 clones produced variable amounts of IFN-γ (Fig. 3).

HLA class II restriction of autoreactive Th clones from PV patients and Dsg3-reactive healthy donors

In light of the preferential detection of HLA-DRB1*0402 and HLA-DQB1*0503 in the studied PV patients and Dsg3-reactive healthy donors, we sought to assess whether these PV-associated HLA class II alleles restricted T cell recognition of Dsg3. Dsg3-reactive Th cell clones from PV patients and Dsg3-reactive healthy donors were cocultured with autologous HLA-DRB1*0402− or

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**FIGURE 1.** Frequency analysis of Dsg3-reactive Th1 and Th2 cells in patients with PV (A) and healthy donors (B). A total of 28 patients with PV and 25 healthy individuals were studied. PBMC from the PV patients (A) and healthy donors (B) were cultured with Dsg3 and x-irradiated, autologous PBMC as APC for 16 h and autoreactive Th1 (IFN-γ-secreting) and Th2 (IL-4-secreting) cells were isolated and quantitated by MACS secretion assay. Patients with acute-onset, chronic active, and remittent PV exhibited both Dsg3-reactive Th1 and Th2 responses (A). In contrast, healthy carriers of the PV-associated HLA class II alleles, HLA-DRB1*0402 (Co16–18) and DQB1*0503 (Co18–25) mounted exclusively Th1 responses against Dsg3 (B). Note that the healthy donor C18 was HLA-DRB1*0402- and HLA-DQB1*0503-positive (+). Healthy carriers of HLA class II alleles other than HLA-DRB1*0402 and HLA-DQB1*0503 (Co1–15) did not exhibit Dsg3-reactive Th responses (B).

**FIGURE 2.** Relationship between the frequency of Dsg3-reactive Th1 and Th2 cells and the autoAb profile of the patients with PV. The mean frequency of Dsg3-responsive Th1 and Th2 cells in the investigated 28 patients with acute-onset, chronic active, and remittent PV as determined by the MACS secretion assay (Fig. 1A) is expressed as box plots (A). While the frequency of Dsg3-reactive Th2 cells was similar in all studied groups of PV patients, the frequency of Dsg3-reactive Th1 cells varied significantly between the groups of patients (p = 0.03), being elevated in the group of patients with chronic active PV (A). There was a direct relationship between the ratio of Dsg3-reactive Th1 and Th2 cells and the levels of Dsg3-reactive IgG1, IgG1, and IgG4 as determined by correlation analyses (r = 0.5; p < 0.0001) for all parameters (B).
HLA-DQB1*0503+ B-LCL as APC and Dsg3. T cell clones 1–5 from DRB1*0402 PV patients PV10 and PV18 were stimulated by Dsg3 in the presence of DRB1*0402 homozygous B-LCL as APC (Fig. 4A). In addition, T cell clones 3–5 were stimulated by Dsg3 with DQB1*0503 homozygous B-LCL as APC (Fig. 4A). T cell clones 6–10 from HLA-DQB1*0503+ PV patients PV1 and PV21 were stimulated by Dsg3 with DQB1*0503+ as well as with DRB1*0402 B-LCL as APC (Fig. 4B). Dsg3-responsive T cell clones 11–14 from DRB1*0402 donor Co16 were only stimulated in the presence of DRB1*0402 B-LCL as APC (Fig. 4C). Similarly, T cell clones 15–18 from DQB1*0503+ donors Co23 and Co24 were stimulated only with DQB1*0503 B-LCL as APC (Fig. 4D).

To further define the immunogenetic HLA restriction of T cell recognition of Dsg3, activation of autoreactive T cells by Dsg3 and HLA-matched homozygous B-LCL was analyzed in the presence of mAb against HLA-DR, -DQ, and -DP. The proliferative responses to Dsg3 of the T cell clones with HLA-DRB1*0402 or HLA-DRB1*0503 homozygous B-LCL as APC are shown in Fig. 4. In the PV patients, two distinct restriction patterns were identified. The proliferative response of the Dsg3-reactive T cell clones 1–5 from DRB1*0402+ PV patients PV10 and PV18 that recognized Dsg3 with DRB1*0402+ (Fig. 5A) or DQB1*0503+ (Fig. 5B) B-LCL as APC was almost completely inhibited by anti-DR (Fig. 5A) and anti-DQ Ab (Fig. 5B), respectively. Accordingly, the proliferative response of the Dsg3-reactive T cell clones 6–10 from DQB1*0503+ PV patients PV1 and PV21 that recognized Dsg3 with DRB1*0402+ (Fig. 5C) and DQB1*0503+ (Fig. 5D) B-LCL as APC was almost completely inhibited by anti-DR (Fig. 5C) and anti-DQ Ab (Fig. 5D), respectively. In contrast, Dsg3-reactive Th1 clones 11–14 from HLA-DRB1*0402+ healthy donor Co16 were only stimulated by Dsg3 with DRB1*0402 homozygous B-LCL as APC, and their proliferative response was blocked by anti-DR Ab (Fig. 5E). Dsg3-reactive Th1 clones 15–18 from HLA-DQB1*0503+ healthy donors Co23 and Co24 were stimulated by Dsg3 only with DQB1*0503 homozygous APC, and their proliferative response was blocked by anti-DQ Ab (Fig. 5F).

**Discussion**

The purpose of this study was to quantitate and characterize autoreactive Th1 and Th2 cell responses against Dsg3 in 28 clinically well-characterized patients with pemphigus vulgaris and in 25 healthy control donors. Both Dsg3-autoreactive Th1 and Th2 cells were isolated from patients with acute-onset, chronic active, and remittent PV. The appearance of Dsg3-reactive Th2 was constant at the different disease stages, while Dsg3-reactive Th1 cells were detected at a significantly higher frequency in chronic active PV. The ex vivo isolated IFN-γ- and IL-4-secreting Dsg3-reactive T cells were indeed of the Th1 and Th2 types, respectively, because they retained a polarized cytokine profile upon long term in vitro

![Image](http://www.jimmunol.org/Downloadedfrom http://www.jimmunol.org)
culture. The selection of Th1 and Th2 cells was specific for Dsg3-responsive T cells, since we were unable to isolate either Th1 or Th2 cells from PBMC of Dsg3-unresponsive healthy donors that had been cultured in vitro with Dsg3 for 16 h before MACS secretion assay (Fig. 1B). This observation clearly demonstrates that both autoreactive Th1 and Th2 cells are present in PV, although at different frequencies during the course of the disease. These findings extend previous studies that derived both Dsg-reactive Th1 and Th2 clones from the peripheral blood of patients with PV (11–14, 23). Their detection, however, requires assay systems of adequate sensitivity, as demonstrated herein compared with ELISPOT analysis (16).

In this report we demonstrate that T cell recognition of Dsg3 in several PV patients and Dsg3-responsive healthy controls is restricted by the PV-associated HLA class II alleles, DRB1*0402 and DQB1*0503. We found that only APC expressing HLA-DRB1*0402 and HLA-DQB1*0503 were capable of presenting Dsg3 to autoreactive Th1 and Th2 clones and that their proliferative response was blocked by anti-DR and anti-DQ Ab, respectively. Therefore, HLA-DRB1*0402 and HLA-DQB1*0503 appear to be the major HLA class II alleles involved in restriction of T cell responses against Dsg3 peptides, thus confirming previous evidence of DRB1*0402 as a restriction element for the presentation of Dsg3-derived peptides (11, 12, 23).

Some of the autoreactive Th clones from the HLA-DRB1*0402+ PV patients were also stimulated by Dsg3 in the presence of both DRB1*0402+ and DQB1*0503+ APC, and Th clones from the HLA-DQB1*0503+ PV patients were also stimulated by Dsg3 in the presence of both DQB1*0503+ and DRB1*0402+ APC. These findings strongly suggest that autoreactive T cells from PV patients recognize certain (not yet identified) Dsg3 peptides that bind to both DRB1*0402 and HLA-DQB1*0503. It is noteworthy that both HLA class II alleles share a negative charge at a critical peptide binding site. Moreover, a recent study from our laboratory showed in healthy donors that Dsg3 peptides may also be presented by HLA class II alleles that share distinct peptide binding motifs with the PV-associated HLA-DRB1*0402 or DQB1*0503 alleles. Dsg3-reactive T cell clones from a Dsg3-reactive healthy donor were restricted by HLA-DRB1*1102 that shares the negatively charged residues, asparaginic acid and glutamic acid, at positions DRβ70 and β71 with the PV-associated allele, HLA-DRB1*0402, and by HLA-DQB1*0301 that shares asparaginic acid at position B57 with the PV-associated HLA-DQB1*0503 (14, 23). The importance of these distinct peptide binding motifs of the aforementioned HLA class II alleles is supported by the identification of Dsg3 peptides, such as peptide DG190 to DB190 in P pocket that may be critical for binding to the negatively charged P4 pockets of DRB1*0402 (DRβ70 and -71) and DQB1*0503 (DQβ57) (17, 25). The ongoing identification of immunodominant T cell epitopes of Dsg3 will help to address the question of whether some Dsg3 peptides promiscuously bind to both DRB1*0402 and DQB1*0503.

Of particular interest is the exclusive detection of Dsg3-reactive Th1 cells in healthy carriers of the PV-associated HLA class II alleles, HLA-DRB1*0402 and HLA-DQB1*0503. In addition, Dsg3-reactive Th1 clones derived from these Dsg3-reactive healthy donors were indeed restricted by HLA-DRB1*0402 and DQB1*0503, respectively. This finding strongly suggests that T cell recognition of Dsg3 in PV patients and healthy individuals depends on the presentation of Dsg3 peptides by distinct HLA class II alleles independent from the development of PV. This observation extends a previous study from our group that detected...
peripheral T cell responses to Dsg3 in a subset of healthy donors (13). This finding is not unexpected, since T cells specific for the autoantigens of multiple sclerosis, myelin basic protein (MBP) and proteolipid protein, have been also identified in healthy individuals and in the cord blood of newborns (26–28). A recent study demonstrated that monozygotic twins discordant for systemic sclerosis both carried topoisomerase-specific, autoreactive T cells (29). Autoreactive T cells from the twin with systemic sclerosis and the healthy brother were able to promote the production of topoisomerase-specific autoAb by B cells from the diseased twin, suggesting that lack of peripheral B cell tolerance was critical for the development of the disease. The frequencies of Dsg3-autoresponsive Th cells in PV patients and Dsg3-reactive healthy individuals found in the present study are comparable to those of MBP-reactive T cells (~1/5000 T cells and 1–10/10^6 PBMC, respectively) in patients with multiple sclerosis and MBP-reactive healthy donors (30, 31). Moreover, autoreactive T cells specific for pyruvate dehydrogenase were detected at a frequency of 4.5–8×10^5 PBMC in the peripheral blood of patients with primary biliary cirrhosis by HLA class I tetramer staining (32). Thus, the frequencies of autoreactive Th cells specific for major human autoantigens seem to be in a similar range in both patients and autoantigen-reactive healthy individuals.

The findings of this study strongly suggest that there is a direct relationship between the frequency of autoreactive Th1 and Th2 cells and the titers of Dsg3-reactive IgG1 and IgG4 Ab. Moreover, in chronic PV, the frequency of Dsg3-reactive Th1 cells was significantly increased. This Th1 predominance is paralleled by a switch from the Th2-regulated IgG4 to the Th1-regulated IgG1 Dsg3-reactive autoAb in chronic PV as shown by previous studies (4, 24). Since all patients with chronic PV received systemic glucocorticoids and immunosuppressive agents, the observed Th1 increase may have been biased by the therapeutic regimen. This seems unlikely in light of a recent serological study showing that there is, rather, a decrease in Th1 cytokines (IFN-γ, TNF-α) in patients with Graves’ disease upon systemic treatment with glucocorticoids (33). Moreover, an independent study with ragweed- and tetanus toxoid-specific human Th1 and Th2 clones showed that corticosteroids had a suppressive effect on both Th1-derived (IFN-γ) and Th2-derived (IL-4, IL-5, IL-13) cytokine expression (34).

A synergistic interplay of autoreactive Th1 and Th2 cells seems to be critical for promoting IgG1 and IgG4 secretion by Dsg3-reactive B cells in the pathogenesis of PV. This is supported by the finding that neither the frequency of Dsg3-reactive Th1 cells nor the presence of Dsg3-specific IgG autoAb, respectively. Thus, even though Dsg3-reactive Th2 cells (which were restricted to the PV patients) are presumably critical for the development of PV, the simultaneous presence of both Th1 and Th2 cells related best to the titers of autoAb against Dsg3. Similarly, in patients with systemic sclerosis (scleroderma), both topoisomerase-specific Th1 and Th2 cells synergistically activated autologous B cells to produce topoisomerase-specific autoAb (35); T cell help for Ab production was strictly dependent on the secretion of IL-2 and IL-6 (35). A similar observation was made in patients with systemic lupus erythematosus. Histone-specific autoreactive Th1 and Th0 cells were able to induce the secretion of anti-histone or anti-DNA autoAb upon in vitro coculture with autologous B cells (36). Autoreactive Th1 cells with a conserved TCR were identified in myasthenia gravis, suggesting a role for these cells in the pathogenesis of this autoimmune disorder (37, 38). However, there is only proof for a critical role of autoreactive Th2 cells in promoting anti-acetylcholine-specific autoAb production in myasthenia gravis (38). Our findings together with the mentionioned in vitro studies strongly suggest that autoAb production in PV depends on both autoreactive Th1 and Th2 cells. We hope that ongoing in vitro coculture studies of autoreactive Th and B cells will help to clarify the role that these Th subsets play in the maintenance of autoimmunity vs tolerance against Dsg3.

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