Type I Regulatory T Cells Specific for Desmoglein 3 Are More Frequently Detected in Healthy Individuals than in Patients with Pemphigus Vulgaris

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Type I Regulatory T Cells Specific for Desmoglein 3 Are More Frequently Detected in Healthy Individuals than in Patients with Pemphigus Vulgaris

Christian Veldman, Annette Höhne, Detlef Dieckmann, Gerold Schuler, and Michael Hertl

Pemphigus vulgaris (PV) is a severe autoimmune bullous skin disorder and is primarily associated with circulating autoantibodies against desmoglein 3 (Dsg3) that are presumably regulated by Th cells. The aim of this study was to identify Dsg3-specific T regulatory (Tr) cells that may help to maintain and restore natural tolerance against Dsg3. Dsg3-responsive IL-10-secreting Tr1 cells were isolated by MACS cytokine secretion assay from healthy carriers of the PV-associated HLA class II alleles, DRB1*0402 and DQB1*0503, but were only rarely detected in PV patients. The Dsg3-specific Tr1 cells secreted IL-10, TGF-β, and IL-5 upon Ag stimulation, proliferated in response to IL-2 but not to Dsg3 or mitogenic stimuli, and inhibited the proliferative response of Dsg3- and tetanus toxoid-responsive Th clones in an Ag-specific (Dsg3) and cell number-dependent manner. Moreover, their inhibitory effect was blocked by Ab against IL-10, TGF-β, and paraformaldehyde fixation. These observations strongly suggest that 1) Dsg3-responsive Tr1 cells predominate in healthy individuals, 2) their growth requires the presence of IL-2, and 3) they exert their Dsg3-dependent inhibitory function by the secretion of IL-10 and TGF-β. Because autoaggressive T cells responsive to identical epitopes of Dsg3 were recently found both in PV patients and healthy individuals, the identified Tr1 cells may be critically involved in the maintenance and restoration of tolerance against Dsg3.

of IL-10 (7), while Th3 cells preferentially secrete the immunosuppressive cytokine, TGF-β. Tr1 cells exist naturally in the human mucosa and maintain intestinal homeostasis against bacterial pathogens (8) and parasites (9) via the production of IL-10 and TGF-β. Similarly, MHC-autoreactive Tr1-like T cell clones (TCC) isolated from the peripheral blood of healthy donors suppressed Ag-specific T cell responses by the secretion of IL-10 and TGF-β (10).

There is evidence that Tr1 cells may indeed act in an Ag-specific manner. In nickel (Ni) allergy, nonallergic subjects carry Ni-specific T cells that fulfill the criteria of Tr1 cells based on their cytokine profile (IL-10, IL-5, IFN-γ, low IL-4) and their ability to suppress the proliferative response of Ni-activated Th1 cells (11) and may thus be critically involved in the down-regulation of Ni-specific Th cell responses in vivo. IL-10+ Tr cells were also detected in patients allergic to bee venom upon speciﬁc immunotherapy with phospholipase A which suppressed the proliferative response of allergen-specific Th cells (12). Moreover, the expression of IL-10 increased during specific immunotherapy with phospholipase A suggesting that the protective effect of this regimen was directly correlated to the presence of IL-10+ allergen-specific Tr cells.

In this study, we assessed whether the presence or absence of Dsg3-speciﬁc Tr1 cells in Dsg3-responsive healthy donors and PV patients, respectively, may be one explanation for the development of tolerance vs autoimmunity against Dsg3. In fact, Dsg3-reactive IL-10-secreting Tr1 cells were identiﬁed in five of six healthy carriers of PV-associated HLA class II alleles (80%) and only in 2 of 12 PV patients (17%) that suppressed the proliferative response of Dsg3-reactive Th cells in an Ag-speciﬁc and cytokine (IL-10/TGF-β) dependent manner. In addition, 50% of the isolated IL-10+ TCC from the healthy donors were of the Tr1 type while only 16% of the IL-10+ TCC from the PV patients were of the Tr1 type. These ﬁndings suggest that Dsg3-speciﬁc Tr may be involved in the maintenance of peripheral tolerance to Dsg3 in healthy individuals and in the restoration of tolerance against Dsg3 in PV patients.

Materials and Methods

Patients and controls

Heparinized blood samples (60 ml) were obtained from a total of 14 adult patients, in treatment at the Dermatology Department (University of Erlangen, Erlangen, Germany), with active and remittent PV as well as from 11 healthy control individuals. All PV patients and healthy control donors gave written consent to participate in this study. 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) the detection of circulating autoantibodies by indirect immunofluorescence microscopy (intercellular IgG binding to epithelial cells of monkey esophagus) and/or by a commercial Dsg3-ELISA (MDL, Naka-ku Nagoya, Japan) (Table I). PV was deﬁned to be active for patients suffering from blisters/erosions on the mucosal surfaces and/or skin; some of these patients had already received immunosuppressive treatment (Table I). Patients with remittent PV had not

Table I. Clinical and immunological proﬁle of the studied patients with PV

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>HLA Class II&lt;sup&gt;a&lt;/sup&gt; Alleles</th>
<th>Clinical Phenotype</th>
<th>Autoantibody Profile&lt;sup&gt;b&lt;/sup&gt; (IgG)</th>
<th>IL-10+ T Cell Clones&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DRB1</td>
<td>DQБ1</td>
<td></td>
<td>Anti-Dsg3</td>
</tr>
<tr>
<td>Active PV</td>
<td></td>
<td></td>
<td></td>
<td>Medication&lt;sup&gt;d&lt;/sup&gt;(per day)</td>
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<tr>
<td>PV2</td>
<td>0401,1401</td>
<td>0301,0503</td>
<td>Skin</td>
<td>Discrete erosions</td>
</tr>
<tr>
<td>PV3</td>
<td>0804,1411</td>
<td>0402,0503</td>
<td>Diss. blisters</td>
<td>Erosions</td>
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<tr>
<td>PV4</td>
<td>0701,1401</td>
<td>02,0503</td>
<td>None</td>
<td>Discrete oral erosions</td>
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<td>PV6</td>
<td>0405,1401</td>
<td>0302,0503</td>
<td>None</td>
<td>Discrete erosions</td>
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<tr>
<td>PV7</td>
<td>0402,0405</td>
<td>02,0302</td>
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<td>Erosions</td>
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<td>PV8</td>
<td>0402,1104</td>
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<td>None</td>
<td>Buccal erosions</td>
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<td>PV9</td>
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<td>0301,0302</td>
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<td>Gingival erosions</td>
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<td>PV10</td>
<td>0803,1405</td>
<td>0503,0601</td>
<td>Blister/erosions</td>
<td>Erosions</td>
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<td>PV11</td>
<td>0402,1104</td>
<td>0301,0302</td>
<td>Discrete erosions</td>
<td>Buccal erosions</td>
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<tr>
<td>PV13</td>
<td>1401,1502</td>
<td>0503,0601</td>
<td>None</td>
<td>Single erosions</td>
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<td>PV14</td>
<td>0301,0402</td>
<td>02,0302</td>
<td>None</td>
<td>Discrete anguinal erosions</td>
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<tr>
<td>Remittent PV</td>
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<td></td>
</tr>
<tr>
<td>PV1</td>
<td>0101,1401</td>
<td>0501,0503</td>
<td>None (none)</td>
<td>None</td>
</tr>
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<td>PV5</td>
<td>0401,0804</td>
<td>0302,0402</td>
<td>None (few blisters on the trunk)</td>
<td>None (buccal erosions)</td>
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<tr>
<td>PV12</td>
<td>0402,1301</td>
<td>0302,0603</td>
<td>None (crusty erosions of the scalp)</td>
<td>None (discrete oral erosions)</td>
</tr>
</tbody>
</table>

<sup>a</sup> As classiﬁed in Materials and Methods.

<sup>b</sup> PV-associated HLA class II alleles (bold).

<sup>c</sup> As determined by ELISA with Dsg3. Values are expressed as index value ([A<sub>405</sub> (Sample) − A<sub>405</sub> (negative control)]/[A<sub>405</sub> (positive control) − A<sub>405</sub> (negative control)]) × 100. Index values $>$14 were considered to be signiﬁcant.

<sup>d</sup> IL-10+ TCC isolated from each patient (Th2, Tr1).
alleles did not. The frequency of the IL-10 responses against Dsg3. In contrast, healthy carriers of other HLA class II alleles did not. The frequency of the IL-10 T cells in healthy carriers of the PV-associated HLA class II alleles was significantly higher compared with those in the PV patients (p < 0.009) and the other healthy donors (p < 0.0021).

Ex vivo isolation and quantification of Dsg3-reactive IL-10-secreting T cells

IL-10-secreting T cells were isolated from short-term (16 h) cultures with 6–9 × 10^6 PBMC of PV patients/controls and 10 μg/ml Dsg3 by MACS cytokine secretion assay following the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Upon termination of the cultures, high affinity anti-human IL-10 Ab which bound to the surface of the cytokine-secreting T cells was added. Labeling with a secondary magnetic bead-coupled Ab allowed for specific enrichment of IL-10-secreting T cells by passage over magnetic columns that were finally counted in a hemocytometer, as recently described for the isolation of IL-4- and IFN-γ-secreting Dsg3-responsive T cells (4). The number of MACS-isolated T cells was divided by the total number of PBMC to obtain the frequency of IL-10^+ T cells per 10^8 PBMC. The statistical software package SAS (version 8.2; SAS Institute, Cary, NC) was used for descriptive uni- and bivariate statistics.

In vitro propagation of IL-10-secreting T cells

Following isolation by MACS assay, Dsg3-reactive IL-10-secreting T cells were cloned by limiting dilution and were expanded by repeated stimulation with 1% PHA (Life Technologies, Karlsruhe, Germany) and x-irradiated (50 Gy) allogenic PBMC as APC followed by addition of IL-2 (10 U/ml; BD-Boehringer, Heidelberg, Germany) as described recently (4). 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 penicillin, 100 μg/ml streptomycin (P/S), and 20 mM l-glutamic acid (l-Glu). Cloned T cells (5 × 10^5) were cultured in duplicate with Dsg3 (10 μg/ml) or PHA (1%; Sigma-Aldrich, Taufkirchen, Germany) and 5 × 10^5 x-irradiated (50 Gy) autologous PBMC as APC in 200-μl 96-well round-bottom microtiter plates (BD-Falcon, Heidelberg, Germany) for 72 h at 37°C in 5% CO2. In addition, TCC were also stimulated with plate-bound anti-CD3 (clone UCHT1; at 10 μg/ml) plus soluble anti-CD28 (clone CD28.2; at 10 μg/ml) (both from BD PharMingen, Heidelberg, Germany). T cell proliferation was determined by the extent of incorporation of [3 H]thymidine (Dupont, Mechelen, Belgium), which was added for the final 18 h of the culture and was expressed as a stimulation index (SI), which is the ratio of [3 H]thymidine uptake (cpm) in cultures with Ag to the uptake in cultures without Ag; an SI ≥ 3 was considered to represent significant stimulation.

Cytokine profile of IL-10^+ T cells

TCC were stimulated with Dsg3 and autologous x-irradiated (50 Gy) PBMC as APC or anti-CD3/anti-CD28 for 48 h and culture supernatants

![FIGURE 1. Frequency analysis of desmoglein 3-reactive IL-10^+ T cells in patients with PV and healthy donors. PBMC from 14 PV patients and 11 healthy donors (controls) were stimulated with Dsg3 for 16 h and IL-10^+ T cells were isolated by MACS secretion assay. Both PV patients and healthy carriers of the PV-associated HLA class II alleles, HLA-DRB1*0402 and DQB1*0503, mounted significant IL-10^+ T cell responses against Dsg3. In contrast, healthy carriers of other HLA class II alleles did not. The frequency of the IL-10^+ T cells in healthy carriers of the PV-associated HLA class II alleles was significantly higher compared with those in the PV patients (p < 0.009) and the other healthy donors (p < 0.0021).

Production and purification of human rDsg3

The recombinant protein PVhis, a fusion protein consisting of the entire extracellular domain of Dsg3 linked to an E tag and histidine tag was used as a source of human Dsg3 and was expressed in a baculovirus system. The recombinant protein PVhis, a fusion protein consisting of the entire extracellular domain of Dsg3 linked to an E tag and histidine tag was used as a source of human Dsg3 and was expressed in a baculovirus system with SF21 insect cells as described previously (4, 6). For the production of Dsg3 protein, 3 × 10^8 High-Five insect cells were inoculated with PVhis baculovirus at a multiplicity of infection of 10. Culture supernatants of baculovirus-infected insect cell cultures were collected after 4 days and Dsg3 protein was purified from culture supernatants over Ni-NTA-linked agarose (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

**Table II.** HLA class II alleles of healthy individuals and derived Dsg3-specific IL-10^+ T cell clones

<table>
<thead>
<tr>
<th>Controls</th>
<th>HLA-DRB1*</th>
<th>HLA-DQB1*</th>
<th>Th2</th>
<th>Tr1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1302, 1501</td>
<td>0602, 0604</td>
<td>n = 3</td>
<td>n = 3</td>
</tr>
<tr>
<td>2</td>
<td>0301, 0401</td>
<td>02, 0302</td>
<td>(C3-13, C3-19, C3-25)</td>
<td>(C3-6, C3-14, C3-21)</td>
</tr>
<tr>
<td>3</td>
<td>0402, 1401</td>
<td>0302, 0503</td>
<td>n = 2</td>
<td>n = 5</td>
</tr>
<tr>
<td>4</td>
<td>1401, 1501</td>
<td>0503, 0602</td>
<td>(C4-4, C4-11)</td>
<td>(C4-2, C4-10, C4-23 C4-28, C4-40)</td>
</tr>
<tr>
<td>5</td>
<td>1501, -</td>
<td>0602, -</td>
<td>n = 6</td>
<td>n = 2</td>
</tr>
<tr>
<td>6</td>
<td>0402, 1501</td>
<td>0303, 0602</td>
<td>(C6-5, C6-13, C6-31, C6-33, C6-39, C6-42)</td>
<td>(C6-9, C6-34)</td>
</tr>
<tr>
<td>7</td>
<td>0402, 1102</td>
<td>0301, 0302</td>
<td>n = 3</td>
<td>n = 4</td>
</tr>
<tr>
<td>8</td>
<td>0101, 1501</td>
<td>0501, 0602</td>
<td>(C7-17, C7-22, C7-42)</td>
<td>(C7-19, C7-27, C7-32, C7-34)</td>
</tr>
<tr>
<td>9</td>
<td>0701, -</td>
<td>02, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1104, 1401</td>
<td>0301, 0503</td>
<td>n = 1</td>
<td>n = 1</td>
</tr>
<tr>
<td>11</td>
<td>1301, 1401</td>
<td>0503, 0603</td>
<td>(C10-6)</td>
<td>(C10-2)</td>
</tr>
</tbody>
</table>

*PV-associated HLA class II alleles (bold).  
IL-10^+ T cell clones derived from each donor.
were analyzed by ELISA for TGF-β immunoreactivity according to the manufacturer’s (BD PharMingen) instructions and by cytometric bead array for IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α immunoreactivities (Th1/Th2 Cytokine CBA 1; BD PharMingen) according to the manufacturer’s instructions.

Flow cytometric analysis
To further characterize the IL-10-secreting TCC, their expression of various surface molecules was compared with Dsg3-specific Th2-like TCC. T cells were immunostained 14–20 days after in vitro stimulation with Dsg3 or PHA with the following Abs: PE- and FITC-conjugated Ab against CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD19 (HB19), CD25 (M-2A5), CD45RO (UCHL 1), CTLA-4 (BN3.1), CD122 (Mik-β2), HLA-DR (G46-6), CCR4 (1G1), CCR5 (2D7), CCR7 (3D12; all from BD PharMingen), TGF-β (TB21; IQ Products, Groningen, The Netherlands), glucocorticoid-induced TNFR (GITR) (N-14; Santa Cruz Biotechnology, Heidelberg, Germany), and respective mouse and rat isotype controls were used. Cells were washed and stained for 30 min at room temperature with optimal dilutions of each Ab, washed again, and analyzed by flow cytometry (FACS Scan and CellQuest Software; BD Biosciences, Heidelberg, Germany).

In vitro regulatory function of IL-10 T cells
IL-10+ TCC were cocultured with HLA-matched autologous Dsg3- and tetanus toxoid (TT)-specific Th2 clones, Dsg3 (10 μg/ml) and autologous, x-irradiated (50 Gy) PBMC as APC. The proliferative response of the “responder” Th2 clones to Dsg3 and the potential extent of inhibition of Th cell proliferation by the IL-10+ TCC was determined by the uptake of [3H]thymidine after 3 days. The proliferative response of the Dsg3-responsive IL-10+ TCC was determined by the uptake of [3H]thymidine after 2 days. To address the critical role of additional suppressive factors secreted by the Tr1 cells, the IL-10+ TCC were also fixed with 2% parramaldehyde for 1 h at 4°C and were subsequently thoroughly washed before use in coculture experiments with responder TCC.

In transwell experiments, a total of 105 cloned Dsg3-responsive Th cells were stimulated with 103 autologous, x-irradiated (50 Gy) PBMC as APC and Dsg3 in 24-well plates; in addition, 2 × 105 Tc cells (1:5) were either added directly to the cultures or 5 × 105 cloned Th cells and 105 Tc cells were placed in transwell chambers (Millicell, 0.4 μm; Millipore, Schwabach, Germany). After 3 days of coculture, T cells were transferred to 96-well plates (5 × 103 cells/well) in triplicate and T cell proliferation was determined by the extent of incorporation of [3H]thymidine, which was added for the final 18 h of the culture.

Table III. Cytokine profile of Dsg3-specific IL-10+ T cell clones

| TCC | IL-2 Ag/Mit | IL-4 Ag/Mit | IL-5 Ag/Mit | IL-10 Ag/Mit | TNF-α Ag/Mit | IFN-γ Ag/Mit | TGF-β/ Ag/Mit | Dsg3 (SI) | Anti-CD3/28 (SI)
<table>
<thead>
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<tbody>
<tr>
<td>Tr1</td>
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</table>

Results
Frequency analysis of Dsg3-reactive IL-10+ T cells in PV patients and healthy donors
The majority (13 of 14) of the studied PV patients expressed either HLA-DRB1*0402 (43%) or HLA-DQB1*0503 (50%), HLA class II alleles prevalent in PV (Table I). Independent of the clinical activity and the immunosuppressive treatment of PV, all of the PV patients exhibited low but reproducibly detectable frequencies of IL-10+, Dsg3-responsive T cells (2.2 ± 0.5/105 cells; Fig. 1). In addition, 11 healthy donors were studied, 6 of whom were either positive for HLA-DRB1*0402 (n = 3) and/or HLA-DQB1*0503 (n = 4; Table II); none of the healthy donors had autoantibodies against Dsg3 as determined by ELISA (not shown). All of the healthy individuals expressing either HLA-DRB1*0402 or HLA-DQB1*0503 carried IL-10+ Dsg3-responsive T cells which were detected at significantly higher frequencies (5.5 ± 1.7/105 PBMC) than that observed for PV patients (2.2 ± 0.9/105 PBMC; p < 0.0009; Fig. 1). Frequencies of IL-10+ Dsg3-responsive T cells of PV patients were similar to the frequencies of IL-4+ Dsg3-responsive T cells reported in a previous study (13). In contrast, Th2 frequencies of healthy donors in the same study were below the detection limit (13). It is noteworthy that none of the healthy carriers of PV-unrelated HLA class II alleles exhibited Dsg3-specific IL-10-secreting T cell responses (Fig. 1).

In vitro expansion of IL-10+ T cell clones
Dsg3-reactive IL-10-secreting T cells were isolated by MACS separation, cloned by limiting dilution and were expanded by repeated stimulation with cells and cytokines as described (13). In contrast, Th2-like TCC were CD4/CD45RO−, GITR+, membrane TGF-β+, produced Th2 cytokines, and proliferated in response to Dsg3 (Table III, Fig. 2). In contrast, the Tr1 cells were positive for CD4/CD45RO+ GITR+, membrane TGF-β+, and did not proliferate in response to Dsg3 (Table III, Fig. 2) (7, 14).
showed a differential expression pattern of distinct surface markers. FACS analysis on the two distinct IL-10/H11001/H9252/GITR and membrane-bound TGF-β expression HLA-DR, CCR4, CCR5, CCR7, and high quantities of CD25, CCR5, CCR7, GITR, and membrane-bound TGF-β. This population was thus classified as Th2 cells (14). These findings were seen with a total of eight Tr1- and six Th2-like TCC.

**Phenotypic analysis of IL-10+ T cells**

FACS analysis on the two distinct IL-10+ T cell populations showed a differential expression pattern of distinct surface markers (Fig. 2). The first CD3+CD4+, memory (CD45RO+) T cell population was negative for CD8, CD14, CD19, and CTLA-4 and expressed HLA-DR and CCR4, but showed no or only marginal expression of CD25, CCR5, CCR7, GITR, and membrane-bound TGF-β. This population was thus classified as Th2 cells (14). These findings were seen with a total of eight Tr1- and six Th2-like TCC.

**Cytokine profile of IL-10+ T cells**

The IL-10+ TCC were stimulated either with Dsg3 and HLA-matched, x-irradiated PBMC as APC or anti-CD3/anti-CD28 for 48 h and culture supernatants were analyzed by ELISA (TGF-β) and cytometric bead array (IL-2, IL-4, IL-5, IL-10, TNF-α). As shown in Table III, two distinct T cell populations were identified. A subset of the IL-10+ TCC failed to proliferate in response to Dsg3 stimulation and produced IL-5, IL-10, TNF-α, and variable quantities of TGF-β upon challenge with Dsg3, resembling Tr1 cells (7). Nonspecific stimulation with anti-CD3/anti-CD28 of the Tr1 cells led to the secretion of IL-5, IFN-γ, TNF-α, and low levels of TGF-β. In contrast, another subset of the IL-10+ TCC which showed a proliferative response to Dsg3 produced substantial quantities of IL-4, IL-5, and IL-10 but no Th1 cytokines upon stimulation with Dsg3 or anti-CD3/CD28 (Table III), resembling Th2 cells (14). Upon stimulation with Dsg3, the Tr1 clones C4-4, C6-13, C7-42 produced IL-10 (25, 40, 23 pg/ml), TGF-β (189, 187, 463 pg/ml), IL-5 (588, 430, 403 pg/ml), and no IL-4 (<10 pg/ml), while the Th2 clones C4-28, C6-34, C6-9, C7-19 produced IL-10 (62, 101, 92, 113 pg/ml), IL-4 (46, 87, 63, 103 pg/ml), IL-5 (267, 545, 633, 329 pg/ml) and no TGF-β (<10 pg/ml).

**Proliferative capacity of IL-10-secreting T cells**

A subset of the IL-10+ TCC that was classified as Tr1 cells based on their cytokine production and phenotype showed no or only little proliferative response to stimulation with Dsg3, PHA, or anti-CD3/CD28 and proliferated only in response to IL-2 (Fig. 3A). In contrast, the Dsg3-responsive IL-10+ Th2 clones showed a significant response to Dsg3 and mitogenic stimulation which was augmented by the addition of IL-2 (Fig. 3B).

**IL-10+ Tr1 cells suppress the proliferative response of Dsg3- and TT-specific Th clones**

To analyze the potential regulatory properties of the IL-10+ Tr1 cells, coculture experiments with Dsg3- and TT-specific responder Th helper clones were performed (Fig. 4). Upon in vitro stimulation with Dsg3, the Tr1 clones significantly inhibited the proliferative response of Dsg3- (Fig. 4A) and TT-responsive (Fig. 4B) TCC.
FIGURE 4. Differential inhibitory function of Dsg3-responsive IL-10$^{+}$ Tr1 and Th2 cells. The IL-10$^{+}$ Tr1 clone C6-13 (A and B) and the Th2 clone C6-9 (C and D) were cocultured with Dsg3- (A and C) or TT- (B and D) specific Th2 clones (C6-34, C3(TT)-10). Dsg3 (10μg/ml) and autologous x-irradiated (50 Gy) PBMC as APC. The proliferative response of the TCC to Dsg3 was determined by the uptake of $[^3H]$thymidine. The proliferative response to Dsg3 of the responder TCC C6-34 was strongly inhibited upon coculture with TCC C6-13 (A) but not C6-9 (C). Accordingly, the TT-dependent proliferation of the TCC C3(TT)-10 was inhibited upon Dsg3-dependent activation of the Tr1 clone C6-13 but not by the Th2 clone C6-9. These findings were seen with a total of six Tr1- and three Th2-like TCC.

This inhibitory effect was cell number-dependent and was detectable up to a Tr1/Th ratio of 1:10 (Fig. 5, A and B); in these experiments, 5 $\times$ 10$^5$ responder Th cells were cocultured with variable numbers (2.5 $\times$ 10$^3$–5 $\times$ 10$^5$) of Tr1 cells. In contrast, Dsg3-responsive IL-10$^{+}$ Th2 clones had no inhibitory effect on the proliferative response of Dsg3- (Fig. 4C) and TT-specific (Fig. 4D) Th clones.

Next, we investigated whether the regulatory function of the IL-10$^{+}$ Tr1 cells was mediated by soluble factors or required cell-cell contact. The inhibitory effect of the IL-10$^{+}$ Tr1 clones on the proliferative response to Dsg3 of a Th2 clone was reversed by blocking Ab against IL-10 and TGF-β and upon fixation of the Tr1 cells (Fig. 5, C and E). In addition, there was a dose-dependent inhibition of the Dsg3-responsive Th2 clones when exogenous rhIL-10 (1–100 ng/ml) or TGF-β (1–100 ng/ml) was added (not shown). Despite the obvious inhibitory action of soluble factors, the additional requirement for close interaction between regulatory and responder T cells could not be excluded. Separation of the two T cell populations in transwell chambers did not abolish the suppressive effect of the Tr1 clones (Fig. 5, D and F). These observations suggest that direct cell contact is not essential for the inhibitory capacity of the IL-10$^{+}$ Tr1 cells, as the semipermeable membrane of transwell chambers allowed free passage of soluble factors, but excluded direct cell-cell contact.

Discussion
In this study, Dsg3-responsive, type 1 T regulatory (Tr) cells were preferentially isolated from the peripheral blood of a subset of healthy individuals who carried the PV-associated HLA class II alleles, HLA-DRB1*0402 and DQB1*0503, and only from a minority of patients with PV. The Tr1 cells exhibited a Dsg3-induced inhibitory action on the proliferative response of Dsg3-responsive, autoreactive Th clones (and also TT-responsive Th clones) which was cell-cell contact independent and was mediated by the cytokines, IL-10, and TGF-β. The predominant isolation of the Dsg3-specific Tr1 cells from the peripheral blood of healthy donors strongly suggests that these Tr1 cells may be involved in the maintenance of self tolerance against Dsg3.

The aim of this study was to address the potential role of autoantigen-specific Tr cells in autoimmunity vs self tolerance against Dsg3 by comparing the presence of Dsg3-specific Tr cells in PV patients and healthy donors. Dsg3-specific T cells secreting the immunoregulatory cytokine, IL-10 and to a lesser degree, TGF-β, were isolated by MACS cytokine secretion assay, cloned by limiting dilution and thoroughly characterized. Our findings demonstrate that a significant portion of the Dsg3-specific IL-10$^{+}$ TCC isolated from healthy donors exhibited characteristics of type 1 Tr cells based on their phenotype, cytokine profile, and in vitro regulatory function. A major finding of the present study was the observation that the immunosuppressive cytokines IL-10 and TGF-β, which were secreted by the Dsg3-specific Tr1 cells, were exclusively responsible for their regulatory function.

There is only limited evidence for the involvement of autoantigen-specific Tr1 cells in autoimmunity (13). A decreased frequency of CD4$^{+}$ T cells producing IL-10, but not IL-2 or IL-4, was observed in rheumatoid arthritis, suggesting a defect in down-regulation of T cell tolerance in this disease (15). In the NOD mouse model of diabetes, both autoantigen-reactive Tr1 cells as well as Th2 cells were induced by immunization with two immunodominant glutamic acid decarboxylase 65 peptides (16). Adoptive transfer of the glutamic acid decarboxylase-reactive Tr1 cells into NOD/scid mice prevented the onset of diabetes. Their mode of action, i.e., cytokine- or cell contact-dependent suppression remained unclear. In patients suffering from multiple sclerosis, oral treatment with myelin basic protein and proteolipid protein clinically induced a state of tolerance associated with a significant increase of myelin basic protein- or proteolipid protein-specific T cells, which secreted predominately TGF-β and moderate quantities of IL-4 and IL-10, which were accordingly classified as Th3 cells (17). Based on the differential secretion of TGF-β and IL-10, respectively, Th3 and Tr1 cells presumably represent two different Tr cell subsets (13). IL-10$^{-/-}$ mice develop colitis and are susceptible to a condition resembling rheumatoid arthritis (18), indicating that this cytokine has an essential role both in maintaining intestinal tolerance to normal enteric Ags and in systemic tolerance
the action of TGF-β on T cells is critical for prevention of autoimmunity, as demonstrated in mice genetically engineered to express a dominant-negative TGF-β receptor II subunit specifically in T cells (19). These mice developed a spontaneous autoimmune disease, with inflammatory infiltrates in several organs and circulating autoantibodies.

Apart from their regulatory action on autoaggressive Th cells responsive to Dsg3, the generated IL-10+ TCC expressed a distinct phenotype characteristic for Tr1 cells. In contrast to the IL-10+ Th2 cells, they were clearly positive for two markers found on regulatory T cell subsets, i.e., GITR and membrane-bound TGF-β. GITR is a cell membrane receptor associated with the regulatory function of CD4+CD25+ T cells and anti-GITR Ab abrogate their regulatory function (20, 21). Even though GITR is also expressed on activated Th cells, there is evidence that Tr cells can be activated through GITR leading to a loss of tolerance in vivo (20). In an animal model of inflammatory bowel disease, both GITR+CD4+CD25+ as well as GITR+CD4+CD25+ T cells act...
as suppressors of inflammation (22). Further studies are required to address the question as to whether activation of the identified Dsg3-specific Tr1 cells through GITR enhances their regulatory capacity or not. Membrane-bound TGF-β may be also an important effector for cell contact-dependent inhibition of CD4+ /CD25+ T cells (23). In contrast, CTLA-4, which is also found on CD4+ /CD25+ Tr cells (24), was not expressed by the identified Dsg3-specific Tr1 cells. Because the transcription factor Foxp3 (scurfin) is associated with the regulatory function of T cells, it may be involved in the regulation of GITR and CTLA-4 (25–27). In humans, mutations of Foxp3 induce an autoimmune syndrome characterized by polyendocrinopathy and enteropathy (28).

Tr1 cells, such as the IL-10+ Dsg3-specific Tr cells identified in the present study, can be induced in vitro by stimulation of naive T cells in the presence of IL-10 and IFN-γ (29). They also appear to be induced by repeated Ag stimulation of naive T cells leading to the down-regulation of immune responses following transfer in vitro (30). Similarly, repetitive in vitro stimulation with APC loaded with tumor-associated Ags (31) or in vivo stimulation with APC bound transforming growth factor β (β) in mice may be also important mediators of Dsg3-specific type 1 Tr cells. These findings provide a sound explanation as to why B cell tolerance against Dsg3 exists in healthy individuals who carry autoaggressive T cells reactive to Dsg3 epitopes identical to those recognized by T cells from the PV patients. Thus, Dsg3-responsive Tr1 cells may represent an ideal tool to therapeutically restore Dsg3-specific immune tolerance in PV.

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