Inhibition of the Transcription Factor Foxp3 Converts Desmoglein 3-Specific Type 1 Regulatory T Cells into Th2-Like Cells

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Pemphigus vulgaris (PV) is a severe autoimmune bullous skin disorder and is associated with autoantibodies against desmoglein (Dsg3) that are regulated by Th2 cells. Recently, Dsg3-specific type 1 regulatory T cells (Tr1) were identified that are presumably critical for the maintenance of tolerance against Dsg3 because there is a much lower Dsg3-specific Tr1:Th2 ratio in the PV patients than in healthy individuals. The aim of this study was to down-regulate the transcription factor Foxp3 in Dsg3-specific Tr1 using antisense oligonucleotides because Foxp3 is constitutively expressed by the Dsg3-specific Tr1. Antisense-treated Dsg3-specific Tr1 clones lost expression of Foxp3, glucocorticoid-induced TNFR family-related receptor, and CTLA-4, and started to secrete IL-2, whereas the secretion of IL-5, TGF-β, and IL-10 remained unchanged. Moreover, antisense treatment induced a proliferative response to Dsg3 of the formerly anergic Tr1 and abrogated their suppressor activity on Dsg3-specific Th2 cell clones. Thus, inhibition of Foxp3 mRNA expression in the Tr1 induced a Th2-like phenotype. In conclusion, Foxp3 expression is inherent to Tr1 function, and modulation of Foxp3 expression in autoaggressive Th2 cells may provide a novel therapeutic approach aimed at restoring tolerance against Dsg3 in PV. The Journal of Immunology, 2006, 176: 3215–3222.
autoimmunity in SCID mice (16) and triggers excessive or misdirected immune responses to microbial Ags, causing immunopathology, such as inflammatory bowel disease (IBD), due to hyper-reactivity of the remaining T cells to commensal bacteria in the intestine (18).

Recently, Foxp3, which encodes a transcription factor that is genetically defective in an autoimmune syndrome in humans and mice (19, 20), has been shown to be expressed by professional Treg cells. Furthermore, Foxp3 is required for the development of Treg cells (21–23). Foxp3-deficient mice develop massive autoimmune and IB, which shares many pathogenetic features of mice deficient in CTLA-4 (24, 25) or TGF-β (26, 27). Fontenot et al. (23) showed that adoptive transfer of Treg cells into neonatal mice prevented disease. In addition, mixed bone marrow mice chimeras from Foxp3+ and Foxp3− recipients were healthy. All of the CD4+CD25+ Treg cells in the recipient mice were derived from the Foxp3+ donor. Notably, gene transfer of Foxp3 converts naïve CD4+CD25− T cells toward a regulatory T cell phenotype similar to that of the professional Treg cells (21). Thus, Foxp3 expression is critically required for the development of Treg cells.

Further evidence for a relationship between Foxp3 and Treg cells comes from observations in mice that overexpress a Foxp3 transgene, driven by its own promoter. These mice exhibit various immune system defects, with decreased numbers of CD4+ and CD8+ T cells, diminished in vitro T cell function, and in vivo immunodeficiency (28). Using in vitro assays, Khattri et al. (22) showed that all T cell populations in these mice exhibited Treg activity, including CD4+CD25+ and CD8+ T cells. These experiments show that ectopic expression of Foxp3 can drive the development of Treg cells. Finally, the introduction of Foxp3 in CD4+CD25− T cells via retroviral infection resulted in their conversion into Treg cells (21, 23). These Foxp3-transduced T cells suppressed the activation of uninfected CD4+CD25+ T cells and protected against colitis when transferred into a lymphopenic host together with pathogenic CD4+CD25− T cells. These studies support a model whereby Foxp3 acts to program the development, and subsequent function, of Treg cells.

There is controversial evidence whether Foxp3 is also expressed in T cells other than CD4+CD25+ Tregs. In a recent study, Levings et al. (29) showed that Tr1 lines expressed more Foxp3 mRNA than freshly isolated CD4+CD25− T cells. However, the authors related their finding to the fact that Foxp3 is up-regulated upon activation of human T cells. The same group did not find any Foxp3 mRNA expression in murine Tr1 cell lines (30). A recent study has investigated Foxp3 mRNA expression in human Th1 cell clones (31), showing that Foxp3 mRNA expression was the consequence of activation (CD25high) of this Th cell subset. Activation of mouse CD4−CD25− T cells did not induce Foxp3 expression, showing that its presence in CD4− T cells was not a consequence of cell activation. Neither naive nor activated CD4+CD25+ responder T cells express Foxp3, distinguishing Foxp3 from other Treg-associated molecules (CD25, CTLA-4, and glucocorticoid-induced TNFR family-related receptor (GITR)) that can be acquired by CD4+CD25+ responder T cells once activated. Although the identification of Foxp3 in Treg has greatly expanded our ability to decipher the development and function of this unique population of regulatory T cells, we do not yet fully understand how Foxp3 is controlled.

In this study, we present evidence that Foxp3 expression is an inherent feature of human Tr1 specific for the autoantigen of PV, Dsg3. Foxp3 antigene-driven inactivation of Foxp3 mRNA converted autoreactive, Dsg3-specific Tr1 into Th2-like cells. These Th2-like cells proliferated in response to Dsg3 and mitogenic stimuli and no longer suppressed CD4+ T cell activation. In addition, inactivation of Foxp3 led to loss of Treg-associated phenotypic markers (GITR, CTLA-4) and an increased secretion of the T cell growth factor, IL-2. We show that conversion of Tr1 into Th2-like cells involves reduced Foxp3 mRNA expression, a molecular pathway, which may be exploited to therapeutically restore tolerance against Dsg3 in PV.

Materials and Methods

Production and purification of human recombinant Dsg3

The recombinant protein PVh, 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 expressed in a baculovirus system with SF21 insect cells as described previously (12, 13). For the production of Dsg3 protein, 3 × 106 High-Five insect cells were inoculated with PVh 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 Ni-NTA-agarose (Qiagen) according to the manufacturer’s instructions.

Quantitative PCR of Foxp3 mRNA

Total RNA was extracted from Dsg3-specific T cell clones (TCC) using the RNAeasy kit according to the manufacturer’s specifications (Qiagen). cDNA was synthesized using oligo(dT) primers and SuperScript II Reverse Transcriptase (Invitrogen Life Technologies) according to the manufacturer’s recommendations. Quantitative real-time PCR was performed on an ABI PRISM cycler (Applied Biosystems) using SYBR Green PCR kit (Stratagene), and specific primers optimized to amplify 90–250-bp fragments from the different genes analyzed. A threshold was set in the linear part of the amplification curve, and the number of cycles needed to reach it was calculated for every gene. Relative mRNA levels were determined by using standard curves for each individual gene and further normalization to ribosomal protein S9 (RPS9). Melting curves established the purity of the amplified band. Primer sequences were as follows: Foxp3, 5′-GAA GCC CAT CCG CCA CAA CCT GA-3′ and 5′-CCC TGC CCC CAC CAC CTC TGC-3′; and RPS9, 5′-CCG AGG CAC AGA CGG TGG AA-3′ and 5′-CGA AGG GTC TCC GCC GGG TCA CAT-3′.

In time course experiments, expression of Foxp3 was determined in relation to β-actin by real-time RT-PCR using TaqMan assays on an ABI Prism 7900. Actin primers and probes have been described previously (32). Human Foxp3 primers and probes were purchased as an Assay-On-Demand from Applied Biosystems. Quantity of mRNA was calculated using the ΔΔCT (threshold cycle) method (PE Applied Biosystems; User Bulletin no. 2; ABI PRISM 7700 Sequence Detection System). For each RT-PCR cycle, the CT was determined, being defined as the cycle at which the fluorescence exceeded the baseline by 10 times the standard deviation of the mean baseline emission for cycles 3–10. Foxp3 mRNA levels were normalized to the housekeeping gene β-actin according to the following formula: ΔCT = CTβ-actin − CTFoxp3. Subsequently, Foxp3 mRNA levels were calculated using the ΔΔCT method, i.e., ΔCT values representing mRNA from cells treated with stimulus were set in relation to the ΔΔCT value representing mRNA levels from untreated cells according to the following formula: ΔΔCT = ΔΔCT(treated) − ΔΔCT(un-treated). The relative mRNA level for the respective sample was calculated as 2−ΔΔCT based on the results of control experiments with an efficiency of the PCR of 95%.

Foxp3 antisense phosphorothioate oligonucleotide (ODN)

ODNs complementary to three regions of the human Foxp3 mRNA (Foxp3-AS1-3) that were identified as potential targets for antisense-induced inhibition were synthesized by MWG Biotech with a phosphorothioate backbone. Additionally, a scrambled ODN of Foxp3-AS2 (Foxp3-SCR2) was synthesized as a specific control. The characteristics and sequences of the ODN used are listed (see Table II).

Foxp3 antisense ODN treatment of Dsg3-specific Tr1

A total of three Tr1-like TCC that were recently generated and characterized (15) were cultured overnight in a medium consisting of RPMI 1640 (Invitrogen Life Technologies) with 10% heat-inactivated pooled human serum (PHS; Invitrogen Life Technologies) in 24-well plates (BD Falcon). The next day, culture media were removed, and cells were incubated in medium without PHS (200 μl) containing liposomal ODN complexes at 37°C for 4 h. Liposomal ODN complexes were formed by incubating 10 μg/ml Lipofectamine 2000 (Invitrogen Life Technologies) with different concentrations of ODN added as 100× stock solution for 15 min at room
temperature before addition of T cells. After a 4-h incubation period, dishes were washed twice with PBS, and fresh medium without Lipopolysaccharine 2000 was added.

Proliferative response of Dsg3-specific Tr1
Following Foxp3 antisense ODN treatment, Dsg3-reactive Tr1 were cultured in a medium consisting of RPMI 1640 (Invitrogen Life Technologies) with 10% heat-inactivated PHS (Invitrogen Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin (P/S), and 20 mM L-glutamine. A total of 5 × 10^4 cloned T cells was cultured in duplicate with 5 × 10^4 x-irradiated (50 Gy) autologous PBMC as APC in 200 µl of 96-well round-bottom microtiter plates (BD Falcon) with Dsg3 (10 µg/ml) or PHA (1%; Sigma-Aldrich) 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 obtained from BD Pharmingen). T cell proliferation was determined by the extent of incorporation of [³H]thymidine (DuPont), which was added for the final 18 h of the culture and was expressed as a stimulation index. This is the ratio of [³H]thymidine uptake (cpm) in cultures with Ag without Ag. A stimulation index ≥ 3 was considered to represent a significant stimulation.

Cytokine profile of IL-10-secreting T cells
A total of 5 × 10^4 Foxp3 antisense ODN-treated Tr1 were stimulated with Dsg3 (10 µg/ml) and autologous x-irradiated (50 Gy) PBMC as APC or anti-CD3/anti-CD28 (each at 10 µg/ml) for 48 h, and culture supernatants were analyzed by ELISA for TGF-β, TGF-α, IFN-γ, IL-10, TNF-α, and IL-12. T cells were used as positive control.

Flow cytometric analysis
To further characterize the Foxp3 antisense ODN-treated Tr1, their expression of various surface molecules was compared with Dsg3-specific Tr1 and Th2 cell clones. T cells were immunostained with the following Abs: PE- and FITC-conjugated Ab against CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD19 (HIB19), CD25 (M-A251), CD122 (MiK-β2),CCR7 (3D12; all obtained from BD Pharmingen), and GITR (N-14; Santa Cruz Biotechnology), and respective mouse and rat isotype controls were used. For analysis of intracellular CTLA-4 and Foxp3, cells were fixed and saponin permeabilized (fix/perm solution; BD Pharmingen) for 30 min and stained with PE-conjugated CTLA-4-specific Ab (BNI3.1), Foxp3-specific Ab (ab22510; Abcam), or an isotype control. Cells were washed and stained for 30 min at room temperature with each Ab, washed three times with BD Perm/Wash solution (BD Pharmingen), and then analyzed by flow cytometry (FACScan and CellQuest software; BD Biosciences) as described recently (15). Foxp3 protein expression of the Dsg3-specific Tr1 and Th2 cell clones was measured by intracellular fluorescence microscopy after cytospin preparation of the cells and subsequent saponin pretreatment.

In vitro regulatory function of IL-10-secreting T cells
After Foxp3 antisense ODN treatment, 5 × 10^4 Dsg3-reactive Tr1 were cocultured with 5 × 10^4 HLA-matched autologous Dsg3-specific Th2 clones, Dsg3 (10 µg/ml), and 1 × 10^5 autologous, x-irradiated (50 Gy) PBMC as APC in microtiter plates. The proliferative response of the “responder” Th2 clones to Dsg3 and the potential extent of inhibition of Th cell proliferation by the IL-10^- Tr1 clones was determined after 48 h by the uptake of [³H]thymidine.

Results
Dsg3-specific Tr1 express Foxp3 mRNA
Two populations of IL-10^- Dsg3-specific TCC were analyzed using a quantitative PCR specific for Foxp3 mRNA expression. These IL-10^- populations were classified as Th2-like and Tr1-like T cells, respectively, based on their differential secretion of immunosuppressive and Th2-like cytokines and their biological function as described recently (15). Briefly, all Dsg3-specific Tr1 secreted IL-10, TGF-β, and IL-5 but not IL-2, IL-4, TNF-α, and IFN-γ 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 Th2 clones in an Ag-specific (Dsg3) and cell number-dependent manner. The inhibitory effect of the Tr1 was at least partly mediated by IL-10 and TGF-β but not by cell-cell contact (15). When we analyzed Foxp3 mRNA levels in isolated CD4^CD25^- T cells from pooled PBMC probes, we obtained a Foxp3:RPS9 mRNA ratio of 0.396 ± 0.0376, which was used as an arbitrary cutoff value. As a positive control, we used ex vivo isolated human CD4^CD25^- T cells and obtained a Foxp3:RPS9 mRNA ratio of 1.93 ± 0.07 (Fig. 1A). Based on the set cutoff value, Foxp3 mRNA was detected in the IL-10^- Tr1 but not in the Th2-like population (Table I; Fig. 1A). Moreover, the Foxp3 mRNA-positive Tr1 clones also expressed Foxp3 protein, whereas the Foxp3 mRNA-negative Th2 cell clones did not (Fig. 1D). Foxp3 mRNA expression of the Tr1 was maximally induced within 2 days upon stimulation with anti-CD3/CD28 (Fig. 1B) or Dsg3 (Fig. 1C) and decreased to baseline

FIGURE 1. Foxp3 expression of Dsg3-specific, autoreactive Tr1 and Th2 clones. Isolated human CD4^CD25^- and CD4^CD25^ T cells from pooled PBMC probes were compared together with Tr1 and Th2 TCC for Foxp3 mRNA expression (A). Foxp3 mRNA levels of CD4^CD25^- and CD4^CD25^ T cells were used as an arbitrary cutoff value; Foxp3 mRNA levels from CD4^CD25^ T cells were used as positive control. For the activation study, the Tr1 clones C7-17, C7-42, and C4-40 and the Th2 clones C4-10 and C4-28 were cocultured with plate-bound anti-CD3 plus soluble anti-CD28 (B) or Dsg3 (C) for a period of 4 days. Total mRNA was extracted from the TCC on days 0, 1, 2, 3, or 4, and Foxp3 mRNA was quantitated by PCR as described in Material and Methods. Foxp3 protein expression was measured in a resting Dsg3-reactive Tr1 clone (C7-17) and a resting Dsg3-reactive Th2 cell clone (C4-10) as a cytosin by fluorescence microscopy (D).
Phenotypic analysis of Foxp3 antisense ODN-treated Tr1 affected when liposomal Foxp3-AS2 (16 and Th2 TCC (Table II). Foxp3 antisense phosphorothioate ODNs with lipofectin was reduced by 12 Foxp3 mRNA expression (Fig. 2). Cell viability upon treatment was reproducibly inhibited by Foxp3-AS2, whereas the two other Foxp3 antisense ODN (Foxp3-AS1 and Foxp3-AS3) led to a less pronounced and less consistent inhibition of Foxp3 mRNA expression. This effect was specific because a scrambled ODN signed that were complementary to distinct nucleotide sequences to block Foxp3 expression in Tr1, three antisense ODN were de-

Specific inhibition of Foxp3 mRNA by antisense ODN
To block Foxp3 expression in Tr1, three antisense ODN were designed that were complementary to distinct nucleotide sequences of Foxp3 cDNA (Table II). Foxp3 expression was significantly and reproducibly inhibited by Foxp3-AS2, whereas the two other Foxp3 antisense ODN (Foxp3-AS1 and Foxp3-AS3) led to a less pronounced and less consistent inhibition of Foxp3 mRNA expression (Table III). This effect was specific because a scrambled ODN complementary to Foxp3-AS2 (Foxp3-SCR2) had no effect on Foxp3 mRNA expression (Fig. 2). Cell viability upon treatment with lipofectin was reduced by 12 ± 3% and not significantly affected when liposomal Foxp3-AS2 (16 ± 4%) or Foxp3-SCR2 (17 ± 3%) complexes were added.

Phenotypic analysis of Foxp3 antisense ODN-treated Tr1
By FACS analysis, the CD3⁺CD4⁻ Tr1 expressed low CD25 but substantial amounts of CCR7, intracellular CTLA-4, and GITR (Fig. 3). Upon treatment with Foxp3-AS2, the Tr1 completely lost expression of CTLA-4 and GITR, whereas expression of CD4, CCR7, and CD25 was unaffected (Fig. 3). Identical findings were seen in four additional Dsg3-specific Tr1-like TCC (data not shown). In contrast, Foxp3-SCR2 had no effect on the expression of the aforementioned cell surface markers by the Tr1 (Fig. 3) and Th2 (data not shown) cell clones. Th2 cells did not show altered cell surface marker expression upon treatment with Foxp3-AS2 (data not shown).

Proliferative capacity of Foxp3 antisense ODN-treated Tr1
Tr1 showed no or only little proliferative response to stimulation with Dsg3 (10 μg/ml), PHA (1%), or anti-CD3/CD28 (10 μg/ml).

Table I. Differential expression of Foxp3 by IL-10⁺, Dsg 3-specific Tr1 and Th2 TCC

| TCC | Foxp3:RPS9 ratio | TCC | Foxp3:RPS9 ratio |
|-----|-----------------|-----|-----------------|
| C3–13 | 2.02 ± 0.41 | C4–10 | 0.2 ± 0.0 |
| C3–19 | 1.86 ± 0.24 | C4–28 | 0.26 ± 0.06 |
| C3–25 | 2.13 ± 0.16 | C6–9 | 0.16 ± 0.01 |
| C4–40 | 0.41 ± 0.13 | C6–33 | 0.17 ± 0.07 |
| C6–31 | 0.49 ± 0.05 | C7–19 | 0.03 ± 0.01 |
| C6–39 | 0.83 ± 0.15 | C7–27 | 0.12 ± 0.00 |
| C7–17 | 2.36 ± 0.23 | C10–2 | 0.03 ± 0.02 |
| C7–22 | 0.86 ± 0.18 | P6–2 | 0.11 ± 0.01 |
| C7–42 | 0.58 ± 0.24 | P6–7 | 2.89 ± 0.22 |

* T1 cells secreted IL-10, TGF-β, and IL-5 but not IL-2, IL-4, TNF-α, and IFN-γ upon Ag stimulation. Th2 cells secreted IL-2, IL-4, IL-5, and IL-10 but not TGF-β, TNF-α, and IFN-γ upon Ag stimulation.

Cytokine profile of Foxp3 antisense ODN-treated Tr1
Untreated and Foxp3-AS2-treated TCC were stimulated with Dsg3 and HLA-matched, x-irradiated PBMC as APC and culture supernatants were analyzed for various cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) by cytometric bead assay and by ELISA for TGF-β immunoreactivity. Stimulation with Dsg3 led to the secretion of IL-5, TGF-β, and IL-10 but not IL-2 (Fig. 5), IL-4, TNF-α, and IFN-γ (data not shown) by the Tr1. In contrast, Foxp3-AS2-treated Tr1 TCC started to produce increased amounts of IL-2 upon stimulation with Dsg3, whereas the secretion of other cytokines, such as IL-5, TGF-β, or IL-10, largely remained unaffected (Fig. 5). This increased IL-2 secretion was not observed when the Tr1 were treated with Foxp3-SCR2 as a specific control, suggesting that Foxp3 antisense-treatment enabled the formerly anergic Tr1 to proliferate to Dsg3 and mitogenic stimuli (Fig. 4).

Foxp3 antisense 2-treated Tr1 no longer suppress the proliferative response of Dsg3-specific Th clones
To analyze the effect of Foxp3 antisense ODN treatment on the regulatory properties of the Tr1, coculture experiments with Dsg3-specific responder Th clones were performed (Fig. 6). Upon in vitro stimulation with Dsg3, the Tr1 clones significantly inhibited proliferation only in response to IL-2 (Fig. 4). Upon treatment with Foxp3-AS2, the Tr1 clones showed a significant and antisense dose-dependent increased proliferative response both to Dsg3 and the mitogenic stimuli PHA (Fig. 3) and anti-CD3/CD28 (data not shown). This proliferative response was not observed when the Tr1 were treated with Foxp3-SCR2 as a specific control, suggesting that Foxp3 antisense treatment enabled the formerly anergic Tr1 to proliferate to Dsg3 and mitogenic stimuli (Fig. 4).

FIGURE 2. Specific inhibition of Foxp3 mRNA expression of Dsg3-specific Tr1 clones by phosphorothioate antisense ODNs. The Tr1 clones C7-17, C7-22, and C4-40 were incubated in serum-free medium containing liposomal ODN complexes at 37°C for 4 h. Foxp3 expression was significantly and dose-dependently inhibited by Foxp3-AS2. This effect was specific because a scrambled ODN complementary to Foxp3-AS2 (Foxp3-SCR2) had no effect on Foxp3 mRNA expression. These representative findings were seen with a total of three different Dsg3-specific Tr1 clones.
the proliferative response of Dsg3-specific Th cells (Fig. 6). In contrast, Foxp3-AS2-treatment of the Tr1 clones before coculture with the Th2 cell clones dose-dependently inhibited the regulatory function of the Tr1 (Fig. 6). Pretreatment of the Tr1 clones with Foxp3-SCR2 had no inhibitory effect on their regulatory function on Th cell activation (Fig. 6).

**FIGURE 3.** Foxp3 antisense ODN treatment of Dsg3-reactive Tr1 inhibits expression of GITR and CTLA-4. The Dsg3-reactive Tr1 clone (C7-17) constitutively expressed high quantities of GITR, CTLA-4, and CCR7 and low CD25. Upon Foxp3 antisense ODN treatment, GITR and CTLA-4 expression was strongly inhibited, whereas the expression of CD4, CCR7, and CD25 remained unaffected. Treatment of C7-17 with Foxp3-SCR2 had no effect on the expression of these phenotypic markers. These representative findings were seen with a total of four different Dsg3-specific Tr1 clones.

**FIGURE 4.** Foxp3 antisense induces a proliferative response of Tr1 clones to Dsg3 and mitogens. The Tr1 clones C7-17 (A) and C7-22 (B) were cocultured in vitro with Dsg3 or 1% PHA and x-irradiated PBMC as APC. Untreated and Foxp3-SCR2-treated Tr1 clones showed virtually no proliferative response to antigenic or mitogenic stimuli as determined by the uptake of [3H]thymidine. In contrast, Foxp3-AS2-treated Tr1 clones showed a dose-dependent by increased proliferative response to Dsg3 and PHA. These representative findings were seen with a total of five Dsg3-specific Tr1-like TCC.
Discussion

Current concepts support the idea that PV, a severe autoimmune bullous disorder induced by autoantibodies against Dsg3, is the consequence of an imbalance between Dsg3-reactive autoaggressive Th2 cells and Tr1 that may be critical for the maintenance of tolerance against Dsg3 (15). Therefore, our group has put major emphasis on characterizing the role of the transcription factor, Foxp3, which is considered to be directly linked to the suppressor function of Treg cells.

In this study, we provide evidence that Foxp3 expression is an inherent feature of human Dsg3-specific Tr1. Dsg3-specific Tr1 clones constitutively expressed Foxp3, whereas Dsg3-specific autoaggressive Th2 cells were Foxp3 negative. Moreover, Dsg3-specific Tr1 could be converted into Th2-like cells by blocking Foxp3 mRNA expression. Inhibition of Foxp3 mRNA in Tr1 by antisense ODN led to a loss of characteristic phenotypic markers associated with Treg function, an increased secretion of IL-2, a proliferative response to stimulation by the autoantigens Dsg3, and loss of suppressor function.

The observation that all of the Tr1 clones expressed Foxp3 mRNA provides further evidence that phenotype and function of human Tr1 is directly linked to Foxp3 expression. Levings et al. (29) found similar results, showing that human Tr1 cell lines expressed more Foxp3 mRNA than freshly isolated CD4+CD25+ T cells. However, the CD4+CD25+ T cells were found to up-regulate Foxp3 mRNA expression upon in vitro activation, which was associated with the expression of CD25. The later observation is in line with a previous study by Walker et al. (33), who also showed that, upon activation, human CD4+CD25+ T cells started to express both Foxp3 mRNA and protein. Noteworthy, this study also showed that Foxp3 mRNA-positive Dsg3-specific Tr1 clones expressed Foxp3 protein, whereas the Foxp3 mRNA-negative Th2 cell clones did not (Fig. 1). Quantitative analysis of Foxp3 expression of the antisense-treated Tr1 clones was performed at the mRNA level because quantitative Foxp3 protein analysis has not yet been established, and recent quantitative analyses had also chosen PCR analysis of Foxp3 mRNA expression as the standard.
approach (29, 30). Foxp3 mRNA expression discrepancy in the Tr1 of the present and previous studies can be explained by the finding of this study that the levels of Foxp3 mRNA largely depended on the activation status of the Tr1 (Fig. 18).

In this study, Foxp3-inhibited Tr1 no longer expressed CTLA-4 and GITR. GITR is a cell membrane receptor associated with the regulatory function of CD4⁺CD25⁺ T cells, and anti-GITR Abs abrogate their regulatory function (34, 35). Although GITR is also expressed on activated Th cells, there is evidence that Treg cells can be activated through GITR leading to a loss of tolerance in vivo (30). In an animal model of IBD, both GITR CD4⁺CD25⁺ as well as GITR CD4⁺CD25⁺ T cells acted as suppressors of inflammation (36). Recently, CTLA-4 was also found on CD4⁺CD25⁺ Treg cells (37) and was shown to be induced only upon TCR stimulation. CTLA-4 inhibits T cell activation by competing with CD28 for ligand binding to block a synergistic co-stimulation and the delivery of inhibitory signals (38–40). The inhibitory function of CTLA-4 is evidenced by the finding that CTLA-4-deficient mice develop a lethal lymphoproliferative disorder at early age (24, 25). These observations indicate that CTLA-4 down-regulates T cell activation and maintains peripheral tolerance in vivo. Because Foxp3 (Scurfin) is associated with the regulatory function of T cells, it may be also involved in the regulation of both GITR and CTLA-4 (21–23). This contention is supported by the finding that murine Foxp3-infected CD4⁺CD25⁺ T cells expressed more CTLA-4 than their uninfected CD4⁺CD25⁻ counterparts (41).

Foxp3-antisense treatment of the Tr1 enabled these cells to respond to the cognate autoantigens with proliferation and secretion of IL-2. Of note, Foxp3 antisense-treated Tr1 secreted higher concentrations of IL-2 than their Foxp3⁺ counterparts. Likewise, CD4⁺CD25⁺ Treg cells are characterized by their inability to proliferate and produce IL-2 upon specific activation. CD4⁺CD25⁺ Treg cells can promote disengagement of the IL-2 signaling pathway in which upstream signaling is uncoupled from downstream cell cycle progression (42); this process is controlled at the DNA level (43). Foxp3 interacts with nuclear factors of activated T cells and NF-κB to repress cytokine gene expression and effector functions of Th cells (44). Thus, Foxp3 may be a yet-unchallenged dominant-acting repressor molecule that abrogates signal transduction by the IL-2 gene in anergic T cells (45). In this study, secretion of other cytokines, such as IL-5, TGF-β, and IL-10, by Dsg3-specific Tr1 clones was unaffected by treatment with Foxp3 antisense. Because Th1-like cytokines, such as IFN-γ and TNF-α, were not produced, the cytokine pattern of the Foxp3 antisense-treated Tr1 was considered to be typical for Th2 cells, and the continued secretion of IL-10 and TGF-β was no longer indicative for their suppressive function.

In this study, Foxp3 antisense-treated Tr1 no longer inhibited the proliferative response of autoimmune Th2 cells to Dsg3. In addition to secretion of the immunosuppressive cytokines IL-10 and TGF-β (15), the Tr1 may at least partly mediate their regulatory function by consumption of IL-2 derived from the Th2 cells. Impaired suppressor function of CD4⁺CD25⁺ T cells has been documented in patients suffering from autoimmune disorders such as multiple sclerosis (46). These findings point to Tr1 cells as promising candidates not only to prevent the induction but also to inhibit ongoing autoimmune disorders. Infection of murine CD4⁺CD25⁺ T cells with Foxp3-encoding retrovirus resulted in a phenotype similar to the naturally occurring CD4⁺CD25⁺ Treg cells (39). Hence, Foxp3-infected T cells were anergic and suppressive in vitro.

The observations of this report point to a transition of Tr1 to a Th2-like phenotype upon inactivation of Foxp3 mRNA. In vivo, there may be a physiological balance between Dsg3-responsive autoimmune Th2 cells and Dsg3-specific Tr1 required for maintenance of peripheral tolerance because a high ratio of IL-10⁺ Th2:Tr1 was seen in the PV patients, whereas a low IL-10⁺ Th2:Tr1 ratio was commonly found in Dsg3-responsive healthy carriers of PV-associated HLA class II alleles (15). Along this line, a recent study in type I diabetes showed a polarized Th1 response in patients with diabetes and a predominant Tr1 response to islet-derived peptides in nondiabetic, HLA-matched control subjects (47). We concluded that development of type I diabetes depends on the balance of autoreactive Th1 and Treg cells.

In Nickel (Ni) allergy, nonallergic subjects carry Ni-specific Tr1 that secrete IL-10, IL-5, IFN-γ, low IL-4, and suppress the proliferative response of Ni-activated Th1 cells (48), which may be critically involved in the down-regulation of Ni-specific Th cell responses in vivo. IL-10⁺ Tr1 were also detected in patients allergic to bee venom upon specific immunotherapy with phospholipase A, which suppressed the proliferative response of allergen-specific Th2 cells (49).

In summary, the present investigation provides evidence that Foxp3 is inherent to the regulatory function of human Tr1. Our present observation extends the association of Foxp3 to Treg function also to the suppressor activity of human Dsg3-specific Tr1. Most interestingly, inhibition of Foxp3 expression promotes the transition of Dsg3-specific human Tr1 to a Th2-like phenotype; the biological function of which remains to be elucidated. Thus, our findings provide additional information on how the development of Tr1, which are presumably involved in maintaining peripheral tolerance against autoantigens, such as Dsg3, may be regulated. These findings may be extremely helpful for the development of novel approaches aimed at modulating Treg cell function for therapeutic intervention in PV and other autoimmune disorders.

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Disclosures

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