T Regulatory Cells and TH17 Cells in Peri–Silicone Implant Capsular Fibrosis

Dolores Wolfram, M.D.
Evelyn Rabensteiner, M.D.
Cecilia Grundtman, Ph.D.
Günther Böck, Ph.D.
Christina Mayerl, Ph.D.
Walther Parson, Ph.D.
Giovanni Almanzar, Ph.D.
Carlo Hasenöhrl, M.D.
Hildegunde Piza-Katzer, M.D.
Georg Wick, M.D.
Innsbruck, Austria

Background: The authors investigated the immunological mechanisms underlying extensive peri–silicone implant capsule formation, one of the most frequent postoperative complications in patients receiving silicone mammary implants.
Methods: The authors studied immune response activation by phenotypic and functional characterization of lymphocytes accumulated within this tissue. Intracapsular lymphoid cells and autologous peripheral blood mononuclear cells were isolated and analyzed by flow cytometry. The proportion of T regulatory cells ($CD4^+CD25^{high}Foxp3^+CD127^-$), the cytokine profiles, and the T cell receptor repertoire of these cells were examined. Intracapsular T regulatory cells were then further analyzed by immunohistochemistry and functional suppression assays.
Results: In comparison with peripheral blood, the cellular composition of intracapsular lymphocytes showed a predominance of $CD4^+$ cells. Intracapsular T cells predominantly produced interleukin-17, interleukin-6, interleukin-8, transforming growth factor-β1, and interferon-γ, suggesting a TH1/TH17–weighted local immune response. Intracapsular T cells displayed a restricted T cell receptor α/β repertoire. The intact suppressive potential of T regulatory cells was demonstrated in crossover experiments with activated peripheral T cells. They did not, however, suppress intracapsular T cells. Interestingly, ratios of intracapsular T regulatory cells were inversely proportional to the clinical degree of capsular fibrosis.
Conclusion: The authors' results indicate that silicone implants trigger a specific, antigen-driven local immune response through activated TH1/TH17 cells, suggesting that ensuing fibrosis is promoted by the production of profibrotic cytokines as a consequence of faltering function of local T regulatory cells. (*Plast. Reconstr. Surg.* 129: 327e, 2012.)

Local and systemic complications in silicone mammary implants carriers are frequently reported and are controversially discussed in the literature.[1–4] It is generally accepted, however, that the most common complication of silicone mammary implants is capsular contracture, with a reported incidence from 0.5 to 50 percent.[5–10] The exact mechanisms leading to the development of capsular fibrosis are not yet fully known. Previous studies suggested that fibrosis results from a silicone-triggered activation of the immune system[11] mediated primarily by macrophages and CD3/CD4 α/β^+ T cells.[12] These findings suggested that cellular immunity against one or several antigen(s) most probably initiates capsular fibrosis development.[13]

The present study was designed to characterize the phenotype of T cells isolated from peri-implant capsular tissue, to determine their cytokine profile, and to estimate the diversity of the T cell receptor repertoire by spectratyping. In addition, we determined the distribution and activity of T cell subpopulations, namely TH17 effector cells and T reg-

From the Department of Plastic and Reconstructive Surgery, the Laboratory of Autoimmunity, Division of Experimental Pathophysiology and Immunology, Biocenter, and the Institute for Legal Medicine, Innsbruck Medical University; and Excellentbeauty.
Received for publication March 24, 2011; accepted August 3, 2011.
The first two authors contributed equally to this work.
Presented at the Second European Plastic Surgery Research Council Meeting, in Hamburg, Germany, August 26 through 29, 2010, and awarded an invitation to present at the 56th Annual Plastic Surgery Research Council Meeting, in Louisville, Kentucky, April 27 through 30, 2011.

DOI: 10.1097/PRS.0b013e31823aeacf

Disclosure: *The authors have no financial or commercial interest in this study. This study was supported by the Competence Centre Medicine Tirol (K.M.T.) and the Lore and Udo Saldow Donation, Vienna, Austria.*

ulatory cells ($CD4^+CD25^{high}Foxp3^+CD127^-$). In humans, T regulatory cells represent 2 to 3 percent of total $CD4^+$ cells in the peripheral blood.[14] They are essential for maintaining peripheral tolerance, as they can suppress activation and effector functions of autoreactive T cells.[15–20] TH17 cells represent a more recently discovered subset of $CD4^+$ T effector cells, identified by their ability to produce interleukin (IL)-17A, IL-17F, tumor necrosis factor-α, and IL-22.[21–23] Stimulation of TH17 differentiation by IL-6 and transforming growth factor (TGF)–β1, a profibrotic cytokine, places this cell lineage in close proximity to T regulatory cells, as TGF-β1 also induces the differentiation of naïve T cells into T regulatory cells.[24–27] To our knowledge, this is the first contribution to the clarification of their interplay in fibrotic processes caused by silicone implants, such as capsular fibrosis.

PATIENTS AND METHODS

Patients

Peri–silicone mammary implant capsular tissue ($n = 37$) was obtained from 33 women (mean age, 42 years; range, 20 to 61 years) undergoing implant change or removal due to capsular fibrosis, implant deflation, or aesthetic reasons. Patients with silicone mammary implants after breast cancer surgery were excluded from this study, as their immune system might have been altered due to either the disease itself or associated therapies. None of the patients had a history of a chronic inflammatory or autoimmune connective tissue disease. Two of the patients had undergone breast reconstruction after complete mastectomy, one for prophylactic reason due to an elevated familial carcinoma risk and the other because of fibrous cystic mastopathy. In 26 patients, implants were silicone gel-filled, and in seven, they were filled with saline. According to the Baker scoring system, the extent of capsular fibrosis was clinically classified from I to IV.[28] The implant change was performed via a submammary ($n = 28$), periareolar ($n = 1$), or axillary approach ($n = 4$), depending on the scar location from previous operations (Table 1). The study was approved by the local ethics committee (study no. AN2218), and all participants gave their written consent. Because the amount of capsular tissue was limited, not all described experiments could be performed in each single patient.

Isolation and Expansion of Intracapsular T Cells

The capsular tissue was cut into small pieces under sterile conditions and incubated (37°C, 5% CO_2) in a Petri dish in 1640 Roswell Park Memorial Institute medium (Cambrex, Baltimore, Md.) supplemented with 10% fetal calf serum, 1% penicillin, streptomycin, and L-glutamine; 20 U/ml of IL-2 (Sandoz Novartis Research Institute, Vienna, Austria) was added to the culture on days 1 and 3. On day 6, the supernatant was filtered through a 100-μm cell strainer (Becton Dickinson, Bedford, Mass.). The resulting cell suspension was transferred into flat-bottomed 24-well plates and stimulated with 20-ng/ml muromonab-CD3 (OKT3, Janssen-Cilag, Saunderton, United Kingdom) and autologous, irradiated (30 Gy) peripheral blood mononuclear cells. Cell culture medium and IL-2 were refreshed on days 6 and 9. Phenotypic and functional analysis of expanded intracapsular T cells was performed on day 12 of culture.

Generation of Peripheral T Cell Lines

Peripheral blood from patients was obtained on the date of surgery. Peripheral blood mononuclear cells were isolated by density centrifugation over Ficoll Paque (Amersham, Buckinghamshire, United Kingdom). T cell lines were grown as described above. Phenotypic and functional analyses were performed on day 12 of culture.

Phenotypic Profile of T Cell Lines

Expression of cell surface molecules of the T cell lines ($n = 12$) was determined by flow cytometry; 3×10^5 T cells were incubated at 4°C for 60 minutes with appropriate dilutions of directly labeled monoclonal antibodies (CD3-FITC, CD4-PE, CD16-FITC, CD25-FITC, CD45RA-FITC, and CD56-PE from Exbio, Prague, Czech Republic; CD8-PECy7 and CD28-APC from Becton Dickinson, Franklin Lakes, N.J.; and CD45RO-APC, T cell receptor α/β-APC, T cell receptor γ/δ-PE, and Foxp3-APC from eBioscience, San Diego, Calif.). After washing steps with 0.02% bovine serum albumin/phosphate-buffered saline (pH 7.3), fluorescence intensity was analyzed on a FACSCalibur (Becton Dickinson).

Immunohistochemistry

Immunohistochemistry was performed on acetone-fixed, 4-μm cryostat sections of frozen tissue samples. Endogenous peroxidase and unspecific antibody binding were blocked with 0.3% H_2O_2/phosphate-buffered saline and 6.5 mg/ml of

Table 1. Patient Data

Capsular Tissue	Type of Implant	Surgical Access	Indication for Implant Change	Baker Score	Implant Duration (yr)	Implant Position	Smoker
S53	Silicone gel	SM	CF	Baker II–III	6–10	Submuscular	Yes
S55	Silicone gel	SM	CF	Baker III–IV	>10	Submuscular	No
S56	Silicone gel	SM	Aesthetic	Baker II	>10	Submuscular	No
S57	Silicone gel	SM	Aesthetic	Baker II	1–5	Subglandular	Yes
S58	Silicone gel	SM	CF	Baker III	>10	Subglandular	No
S60	Silicone gel	SM	Aesthetic (dislocation)	Baker II	>10	Subglandular	Yes
S61	Silicone gel	SM	Aesthetic	Baker II	1–5	Submuscular	No
S62	Silicone gel	SM	CF	Baker III	1–5	Submuscular	Yes
S64	Saline	A	Aesthetic (deflation)	Baker II	6–10	Submuscular	No
S65	Silicone gel	SM	CF	Baker II–III	>10	Subglandular	Yes
S66	Silicone gel	P	Aesthetic	Baker II	>10	Subglandular	Yes
S69	Silicone gel	SM	Aesthetic	Baker II–III	1–5	Subglandular	Yes
S70	Saline	SM	Aesthetic	Baker I	6–10	Subglandular	Yes
S71	Silicone gel	A	CF	Baker III	6–10	Submuscular	No
S72 dx	Silicone gel	SM	Aesthetic	Baker I–II	6–10	Subglandular	Yes
S72 sin	Silicone gel	SM	Aesthetic	Baker I–II	6–10	Subglandular	Yes
S73	Silicone gel	SM	CF	Baker III–IV	>10	Submuscular	Yes
S74	Silicone gel	SM	Aesthetic	Baker II	6–10	Subglandular	No
S76	Silicone gel	SM	Aesthetic	Baker II	>10	Submuscular	Yes
S77	Silicone gel	A	Aesthetic	Baker I	>1	Submuscular	No
S78	Silicone gel	SM	Aesthetic	Baker II	1–5	Submuscular	No
S79 dx	Silicone gel	SM	Aesthetic	Baker II	1–5	Subglandular	Yes
S79 sin	Silicone gel	SM	CF	Baker III	1–5	Subglandular	Yes
S80	Saline	SM	Aesthetic	Baker II	>10	Submuscular	Yes
S81	Silicone gel	SM	Aesthetic	Baker I	1	Subglandular	No
S82 dx	Silicone gel	SM	CF	Baker III	6–10	Subglandular	No
S82 sin	Silicone gel	SM	Aesthetic	Baker II	6–10	Subglandular	No
S83	Silicone gel	SM	CF	Baker III	1–5	Subglandular	Yes
S84 dx	Silicone gel	SM	CF	Baker IV	6–10	Subglandular	No
S84 sin	Silicone gel	SM	Aesthetic	Baker II	6–10	Subglandular	Yes
S85	Silicone gel	SM	CF	Baker III	<1	Subglandular	No
S86	Saline	SM	CF	Baker III–IV	6–10	Subglandular	No
S88	Saline	SM	CF	Baker III–IV	6–10	Subglandular	Yes
S89	Silicone gel	SM	Aesthetic	Baker I	>10	Subglandular	Yes
S90	Silicone gel	SM	Aesthetic	Baker I–II	>10	Subglandular	Yes
S91	Saline	A	Aesthetic (deflation)	Baker I–II	>10	Submuscular	No
S92	Saline	SM	Aesthetic (deflation)	Baker I–II	6–10	Submuscular	Yes

SM, submammary approach; CF, capsular fibrosis; A, axillary approach; P, periareolar approach; aesthetic, asymmetry or wish for larger or smaller implants; dx, dexter (right side); sin, sinister (left side).
No patients experienced gel bleeding. None of the patients had diabetes.

NaN3 for 30 minutes and blocking reagent (1096176; Boehringer Mannheim, Mannheim, Germany) supplemented with 10% fetal calf serum and 5% goat serum. Slides were then incubated for 12 hours with the following antibodies: rabbit anti-CD25 (ab61777, 1:50; Abcam, Cambridge, United Kingdom), mouse anti-CD3 (M0835, 1:25; Dako, Glostrup, Denmark), mouse anti-Foxp3 (14-4777, 1:50e; Bioscience, Hatfield, United Kingdom), and mouse anti-CD4 (M0716, 1:30; Dako). After washing in phosphate-buffered saline, the secondary antibody [goat anti-mouse AP, D0486, 1:50 (Dako), or goat anti-rabbit HRP, P0448, 1:50 (Dako)] was applied for 30 minutes. Visualization was achieved by adding Fast Red Naphthol (F4523; Sigma-Aldrich, St. Louis, Mo.) or 2,4-diaminobutyric acid (Sigma-Aldrich). Double stainings were achieved with primary antibodies from different species (rabbit anti-CD25/mouse anti-CD3, rabbit anti-CD25/mouse anti-Foxp3, or rabbit anti-CD25/mouse anti-CD4) and different substrates by subsequently performing the described single staining.[12]

Cytokine Analysis

Supernatant from cell culture of capsular T cells and peripheral blood mononuclear cells was collected after 12 days and stored at −20°C until use. Quantitative cytokine levels (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, interferon-γ, and tumor necrosis factor-α) were determined by a FlowCytomix Bead-Based Multiplexing Assay kit (human TH1/TH2 11plex; Bender MedSystems,

Vienna, Austria) according to the manufacturer's instructions. Analyses were performed by flow cytometry and analyzed using FlowCytomix Pro 2.1 software. The concentration of TGF-β1 (Bender MedSystems) and IL-17 (RayBiotech, Inc., Norcross, Ga.) was determined via enzyme-linked immunoabsorbent assay.

IL-10 and IL-17 were also determined in fresh tissue of five patients ex vivo via polymerase chain reaction to further verify the presence of TH17 cells and T regulatory cells. RNA was isolated from fresh tissue (see "RNA Isolation and T Cell Receptor CDR3 Spectratyping," below), and 1 μg was then retro-transcribed at 37°C. cDNA (1 μg) was amplified by polymerase chain reaction in 5× reaction buffer (1.5 mM $MgCl_2$) containing 0.2 mM dNTP and 50 μM betaine, using 1.25 U of Taq DNA polymerase (Promega, Madison, Wisc.) in a final volume of 50 μl.

Regulatory T Cell Isolation

Purification of peripheral and intracapsular T regulatory cell subsets was performed by magnetic cell sorting using the $CD4^+CD25^{high}CD127^-$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The two-step procedure consisted of depletion of non-$CD4^+$ and $CD127^+$ cells over a LD column (MACS, Miltenyi Biotec), followed by positive selection of $CD4^+CD25^+CD127^-$ T regulatory cells from the pre-enriched $CD4^+$ T cell fraction.

Suppression Assays and Crossover Suppression Assays

Sorted $CD4^+CD25^+CD127^-$ T regulatory cells were cocultured at different ratios (1:1, 0.5:1, 0.25:1, and 0.125:1) with 5×10^3 $CD4^+CD25^-$ T effector cells, 5×10^4 autologous peripheral blood mononuclear cells irradiated at 30 Gy for antigen presentation, and 0.1 μg/ml of OKT3 in 96-well U-bottom plates in Roswell Park Memorial Institute medium 1640 with 7% fetal calf serum. In crossover experiments, autologous T effector cells from peripheral blood were cocultured with T regulatory cells from capsules and vice versa. On day 4, 0.5 μCi of ^{3}H-thymidine (Batch B500, no. TRA120; Amersham, Buckinghamshire, United Kingdom) was added for the last 16 hours. Cells were harvested with a cell harvester (Inotech Biosystems International, Inc., Rockville, Md.). Proliferation was determined by ^{3}H-thymidine incorporation in a beta-counter (25600 Multi Purpose Scintillation Counter; Beckman Coulter, Brea, Calif.) on day 5. The percentage of suppression was determined as follows: 1 − (counts per minute incorporated in the coculture/cpm of T effector cells population alone) x 100 percent.

RNA Isolation and T Cell Receptor CDR3 Spectratyping

Total RNA was extracted from peripheral blood mononuclear cells and intracapsular T cells using total RNA isolation reagent, according to the manufacturer's protocol (Sigma-Aldrich). To exclude a cell culture-induced clonal expansion of T cells, RNA was directly isolated from the capsular tissue and from fresh peripheral blood mononuclear cells ($n = 9$). As previously described, T cell receptor Vβ transcripts were amplified by polymerase chain reaction using a Hot-Start Taq Mastermix kit (Qiagen, Vienna, Austria) and primers (MWG Biotech, Ebersberg, Germany) specific for each of the V/β families (24 variable primers) and a specific primer encoding for the constant region of the β-chain and labeled with the fluorescent dye marker 6-FAM as described by Herndler-Brandstetter et al.[29]

Statistics

The paired t test and the nonparametric Wilcoxon test for paired samples were used for normally and nonnormally distributed data sets, respectively. Statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, Wash.) and SPSS 15.0 software (SPSS Inc., Chicago, Ill.). All values are expressed as mean ± SD; p values less than or equal to 0.05 were considered statistically significant.

RESULTS

Immunohistological Detection of T Regulatory Cells within the Capsular Tissue

Immunohistochemical analysis mainly showed an infiltration by activated T cells ($CD3^+CD25^+$) of the capsular tissue, predominantly located adjacent to the frontier layer abutting the silicone implant, the so-called "pseudosynovium," as previously described.[12] $Foxp3^+/CD25^+$ double staining revealed the presence of intracapsular T regulatory cells (Fig. 1). They were mainly found within the frontier layer among the population of T effector cells and perivascularly within the inner and outer vascular layer.[12] Importantly, the number of T regulatory cells was higher in capsules with less fibrosis, which correlated with the results of fluorescence-activated cell sorter analysis as mentioned below.

Fig. 1. T regulatory cells and activated T effector cells are resident in the capsular tissue. (*Left*) Overview of capsule S69 (explanted from the left breast; Baker score, I to II), stained with a-CD3 Fast Red (FR)/a-CD25 diaminobenzidine (DAB; original magnification, ×100; *ps*, pseudosynovium; an arrowhead marks the silicone implant). (*Second from left, above*) Single staining with a-CD3 FR (original magnification, ×400) and (*Second from left, below*) a-CD25 DAB (original magnification ×400) show perivascular accumulation of T cells. Double staining on consecutive slides reveal (*center, above*) perivascular activated T cells ($CD3^+$-FR/$CD25^+$-DAB) and (*center, below*) T regulatory cells ($Foxp3^+$-FR/$CD25^-$-DAB; original magnification, ×400). (*Second from right, above*) Activated T cells ($CD3^+$-FR/$CD25^-$-DAB; original magnification, ×400) and (*Second from right, below*) T regulatory cells ($Foxp3^+$-FR/$CD25^+$-DAB) among collagenous fibers (original magnification, ×600). (*Right, above*) Mouse IgG1 isotype control (original magnification, ×100) and (*Right, below*) normal rabbit serum (original magnification, ×200). Negative controls were done by omitting primary antibodies (not shown). The *center, above* and *center, below* panels are tests on consecutive sections.

$CD4^+$ and T Regulatory Cells Are Increased within Capsular Tissue

T cells isolated from fibrotic tissue were gated for CD3/CD4 and mainly showed a $CD4^+$ phenotype (80 percent; Fig. 2, *above, left*). Only 18 percent of all T cells were $CD8^+$; thus, the CD4/CD8 ratio (4.6) within capsules was significantly increased when compared with peripheral blood (2.1; $p < 0.0001$). Most of intracapsular $CD4^+$ cells (91 percent) expressed CD45RO, whereas peripheral cells showed an activated/memory status (63 percent; Fig. 2, *below*). We found that most intracapsular T cells expressed T cell receptor α/β (94 percent). A higher percentage of intracapsular T cells, however, expressed T cell receptor γ/δ (5 percent) when compared with peripheral blood (3 percent). Fluorescence-activated cell sorter analysis showed that T regulatory cells were more numerous in the capsular tissue than in periphery ($p < 0.01$; Fig. 2, *above, right*).

Inverse Correlation between Numbers of Intracapsular T Regulatory Cells and Severity of Capsular Fibrosis

Fluorescence-activated cell sorter analysis after 2 weeks of cell culture revealed that T regulatory cells were increased in the capsular tissue compared with the periphery. Values for capsular T regulatory cells averaged 5.2 percent within the $CD3^+$ population as compared with 0.7 percent in the periphery ($p < 0.01$; Fig. 2, *above, right*). In capsules with Baker scores I to II, 8.5 percent of all $CD3^+$ cells expressed a T regulatory cell phenotype, whereas in capsules with scores of III to IV, significantly fewer T regulatory cells were present (1.4 percent; $p < 0.01$; Fig. 2, *center, left and right*). Figure 3 shows the relationship between the degree of capsular contracture and the relative amount of T regulatory cells and T effector cells.

Decreased T Cell Regulation in Capsular Tissue

Freshly isolated T regulatory cells were more effectively suppressive than cultured T regulatory cells. We detected a statistically significant difference in the suppressive capacity of T regulatory cells from capsules of patients with Baker scores I to II compared with capsules of patients with Baker scores III to IV ($p < 0.01$ at a ratio 1:0.25; Fig. 4, *above*). T regulatory cells from capsules with a strong fibrotic reaction lacked effective suppression, whereas those from less fibrotic capsules effectively suppressed intracapsular T effector cells. Crossover suppression assays revealed, however, that intracapsular T regulatory cells from both advanced and less advanced fibrotic capsules had higher suppressive capacity than peripheral ones. Moreover, peripheral T effector cells were suppressed more effectively by intracapsular T regulatory cells than intracapsular T effector cells. Thus, T effector cells from capsules must be considered less susceptible to suppression because intracapsular T regulatory cells suppressed peripheral T effector cells effectively but failed to suppress intracapsular T effector cells (Fig. 4, *be-*

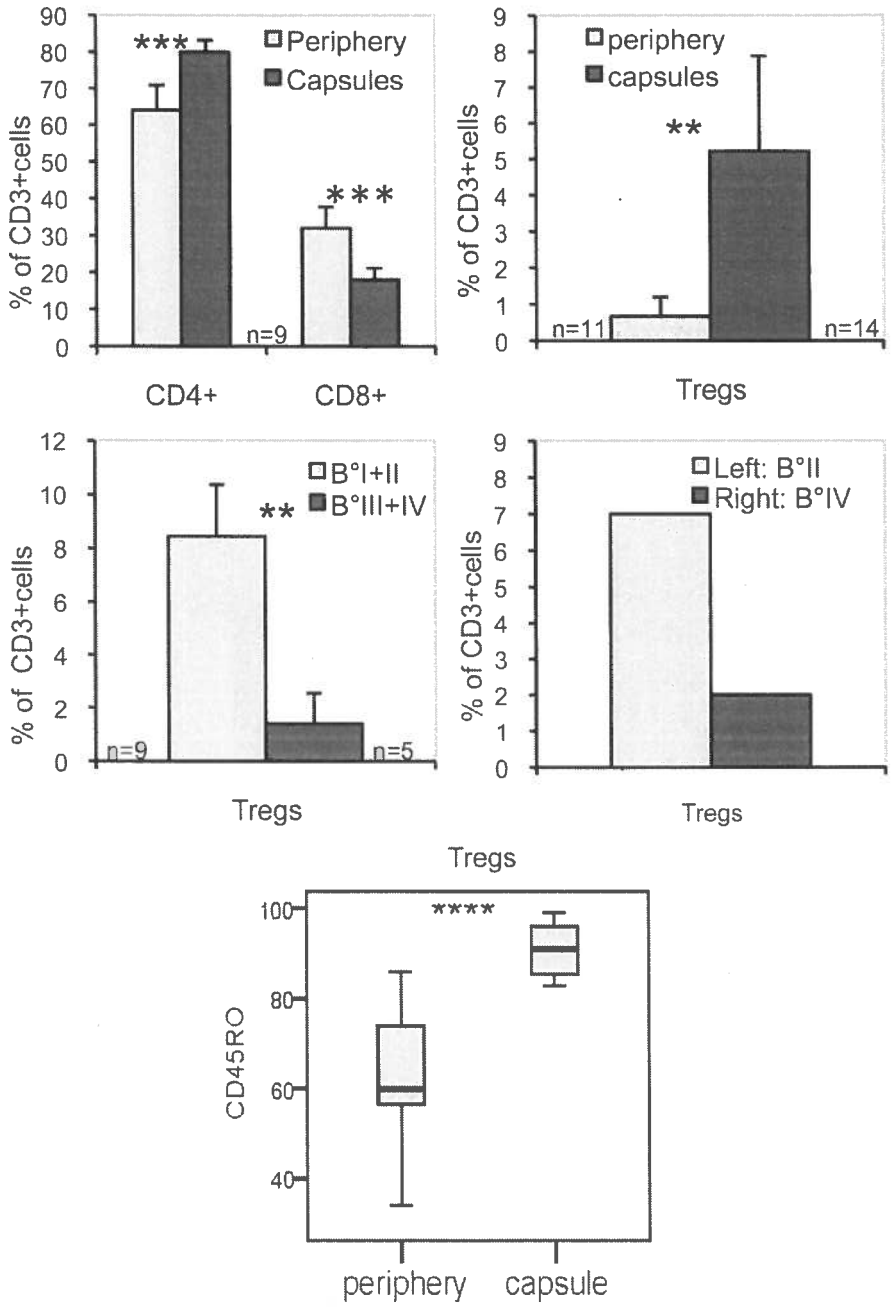

Fig. 2. Composition of the intracapsular T cell population. (*Above, left*) Eighty percent of all intracapsular T cells were $CD4^-$, whereas they made up only 64 percent of peripheral T cells ($p < 0.001$). Peripheral $CD8^+$ cells were more numerous in the periphery (32 percent) as compared with the capsule (18 percent; $p < 0.001$). (*Above, right*) Significantly ($p < 0.01$) higher numbers of $CD4^+CD25^{high}Foxp3^+$ T regulatory cells were found within the fibrotic tissue (5 percent) than in the periphery (0.7 percent). (*Center, left*) Numbers of T regulatory cells correlate inversely with the clinical degree of fibrosis. In capsules with less fibrosis (Baker score I to II), there were significantly more T regulatory cells (8 percent) as compared with capsules with Baker score III to IV (1 percent; $p < 0.01$). (*Center, right*) Numbers of T regulatory cells of the left and right capsule from one patient with different Baker scores on each side (left side, Baker II; right side, Baker IV) correlate inversely with the Baker score (left side, 7 percent; right side, 2 percent). (*Below*) Within capsules, significantly ($p < 0.0001$) higher percentages of CD45RO memory cells were found (capsules, 91 percent; periphery, 63 percent).

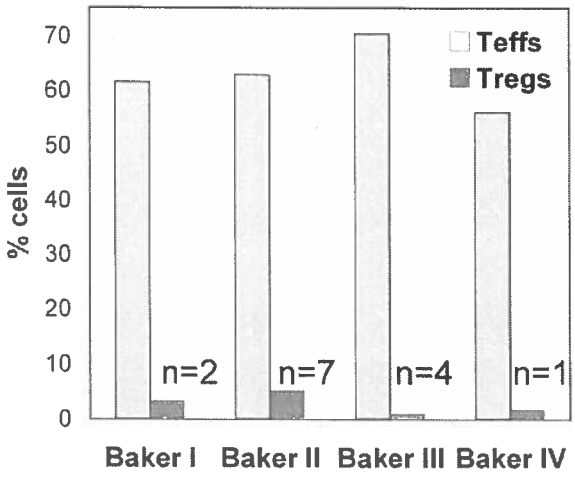

Fig. 3. Graphic representation of the relationship between the degree of capsular contracture and the relative amount of T regulatory cells and T effector cells in the analyzed capsules.

low). The demonstrated TH1/TH17 immune response within the capsular tissue further explains the low susceptibility of T effector cells toward suppression by T regulatory cells.

Peri–Silicone Implant Tissue Is Characterized by a TH1/TH17–Weighted Cytokine Milieu

The most prominent cytokines found in the supernatant of intracapsular mononuclear cells were IL-6, IL-8, IL-17, and interferon-γ, pointing to a TH1/TH17–weighted immune response. Also, IL-1β and tumor necrosis factor-α production, known to be enhanced by IL-17,[24] could be clearly detected in the supernatant of the specimens. Moreover, IL-4 and IL-5, cytokines characteristic for a TH2 response, were less prominent. The profibrotic cytokine TGF-β1 was abundantly found in the supernatant of cultured intracapsular T cells. Compared with intracapsular T cells, significantly lower concentrations in the supernatant of peripheral cells were found for IL-17 ($p < 0.05$), IL-6 ($p < 0.05$), IL-8 ($p < 0.0001$), and TGF-β1 ($p < 0.001$) secretion. The amount of IL-5, however, did not differ significantly ($p \leq 0.085$; Fig. 5, *above*). Interestingly, polymerase chain reaction experiments on ex vivo material revealed the presence mainly of IL-17 and IL-10 in situ (Fig. 5, *below*).

Intracapsular T Cells Have a Restricted T Cell Receptor α/β Repertoire

A total of 66 percent of capsule-derived T cells showed an oligoclonally restricted repertoire compared with 43 percent of peripheral cells. A difference was found in monoclonal T cell expansion (i.e., 23 percent in capsular tissue versus 3 percent

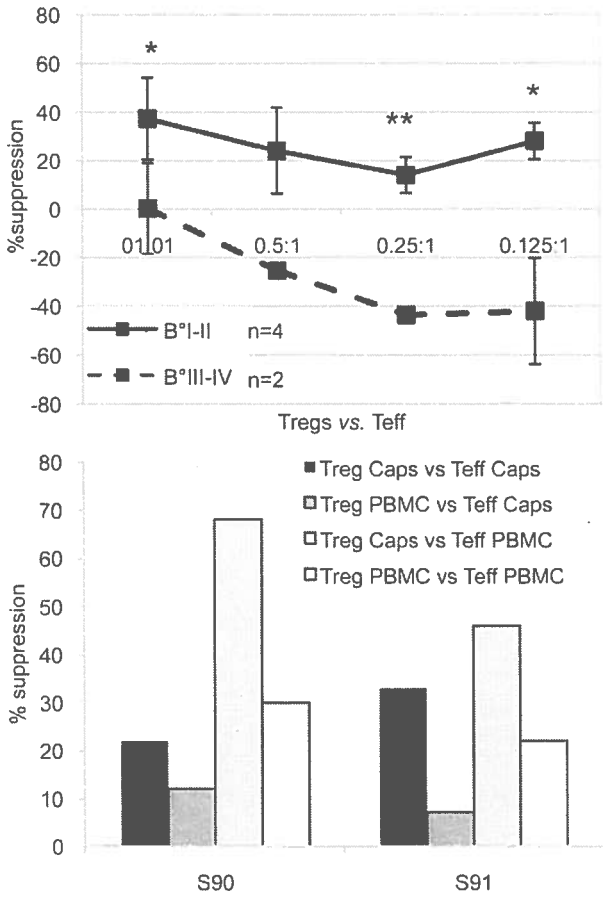

Fig. 4. Suppressive capacity of intracapsular and peripheral T regulatory cells was assessed in suppression assays. (*Above*) T regulatory cells from capsules with less fibrosis suppress intracapsular T effector cells effectively, whereas those from capsules with higher Baker scores fail to achieve effective suppression. (*Below*) Crossover suppression assays in two separate patients reveal the strong suppressive capacity of intracapsular as compared with peripheral T regulatory cells. Intracapsular T regulatory cells, however, suppress peripheral T effector cells (*Treg Caps vs Teff PBMC*) more effectively compared with intracapsular T effector cells (*Treg Caps vs Teff Caps*); *$p \leq 0.05$; **$p \leq 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

in periphery). In the periphery, 54 percent of polyclonal T cell receptor Vβ transcripts with a Gaussian-like distribution were detected, whereas only 9 percent of the capsular T cells showed polyclonal distribution (Fig. 6).

DISCUSSION

Due to the worldwide, steadily growing popularity of silicone mammary implant breast augmentation and the controversies surrounding their overall safety, it is of great diagnostic and clinical importance to clarify the exact molecular

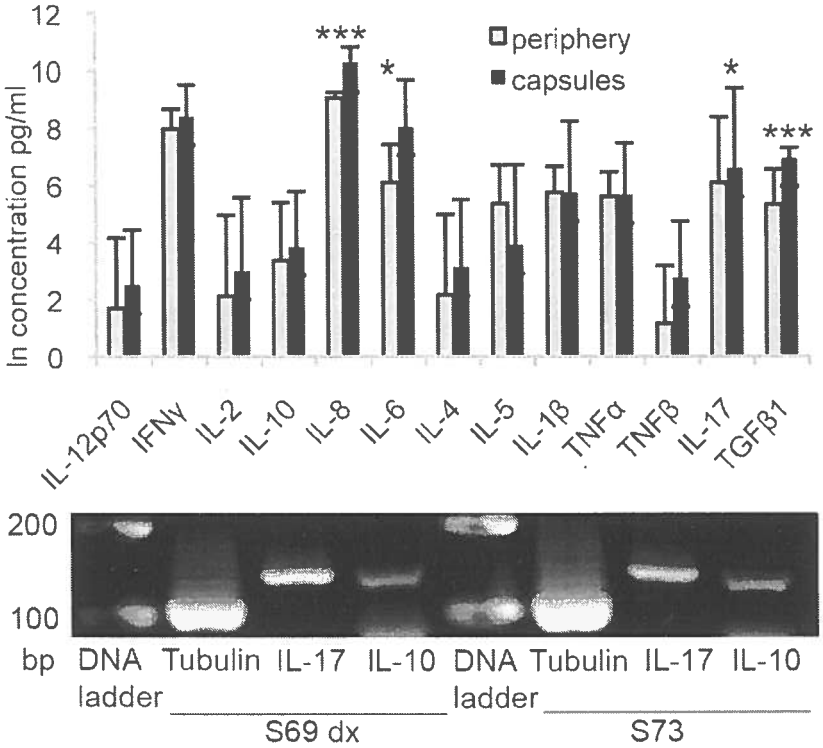

Fig. 5. Cytokine production. (*Above*) Cytokine profile of intracapsular and peripheral cells evaluated from the supernatant. TH1/TH17 cytokines predominate. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$. (*Below*) Detection of IL-17 and IL-10 in two representative capsules by polymerase chain reaction performed with freshly isolated RNA (ex vivo) from capsular tissue (S69 dx, Baker score II; S73, Baker score IV).

mechanisms behind the phenomenon of peri–silicone implant capsule formation. Although the role of T regulatory cells and TH17 cells has extensively been investigated in lung or liver fibrosis, this is the first contribution to clarify their role in capsular fibrosis.[30–33]

The phenotypic analysis of the intracapsular T cells in this study confirmed and extended our previous in situ observations of the presence of $CD4^+$ and $CD8^+$ cells, with a predominance of the TH1 phenotype.[12] T regulatory cells were also more numerous within the capsular tissue as compared with the periphery. Most interestingly, capsules with clinically mild symptoms of capsular contracture (Baker scores I to II) showed a significantly higher number of T regulatory cells in comparison with those with higher stages of fibrosis (Baker scores III to IV). Our data suggest that, at least in an early stage, T regulatory cells play an important role in controlling capsular fibrosis. This effect may be achieved either by down-regulation of T effector cells and/or by imposing a threshold which T effector cells must exceed.

In our crossover suppression assays, we found that T regulatory cells from fibrotic capsules had higher suppressive capacities on peripheral blood T effector cells when compared with peripheral T regulatory cells. Interestingly, suppression assays showed that the suppressive capacity of intracapsular T regulatory cells correlated inversely with the degree of fibrosis. This suggests that either the capsular T effector cells become resistant to suppression by capsular T regulatory cells or decreasing numbers and/or a decreasing suppressive potential of T regulatory cells is no longer able to effectively suppress T effector cells. Thus, it is reasonable to conclude that capsular fibrosis is not merely the result of decreasing numbers of T regulatory cells but a qualitative property of intracapsular, not suppressible, T effector cells. This notion is further supported by the predominance of TH1/TH17 cytokines within the capsules, as TH17 cells are known to be less susceptible to suppression by T regulatory cells.[34–38] Several groups have reported inverse developmental relationship between T regulatory cells and TH17 cells, with IL-6 playing an important role in dictating whether the immune system is dominated by proinflammatory TH17 cells or by protective T regulatory cells.[25–27,39]

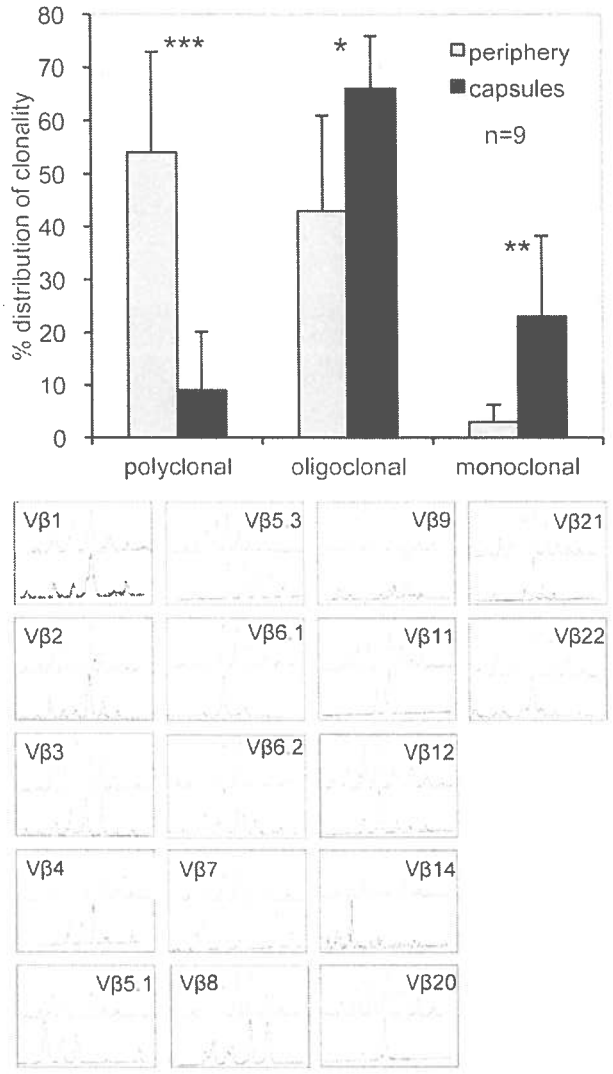

Fig. 6. Intracapsular T cells have a restricted T cell receptor repertoire. (*Above*) Distribution of T cell receptor Vβ transcripts in capsule- and peripheral blood–derived T cells. Mean percentages of the clonal distribution within capsules as compared with the periphery. Significant differences in the polyclonal and monoclonal T cell receptor repertoire were detected. The T cell receptor restriction pattern of Vβ transcripts shows that capsule-derived T cells are generally more restricted than their autologous peripheral counterparts. RNA was isolated from fresh capsular tissue (ex vivo) as well as from fresh uncultivated peripheral T cells. (*Below*) Intracapsular (*lower line*) and peripheral (*upper line*) T cell receptor Vβ transcripts of one representative patient (S57, Baker score II). T cell receptor restriction in capsule-derived cells can be noted. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$.

Furthermore, different groups described functional plasticity of T regulatory cells and TH17 cells, as conversion of T regulatory cells to the TH17-like phenotype may be induced by IL-1β.[40–42] In the supernatant of intracapsular T cells, we found considerable amounts of IL-6 and IL-1β. The released acute-phase proinflammatory cytokines subsequently lead to a local accumulation of intracapsular TH17 cells, probably by converting $CD4^+$ cells and local T regulatory cells into TH17 cells. Tamboto et al. have shown recently that subclinical (biofilm) infection causes capsular contracture in a porcine model following augmentation mammaplasty.[43] They postulate that *Staphylococcus epidermidis*, a predominant member of resident breast flora, gains access to the mammary implant and forms a biofilm on contact with the silicone surface, leading to the formation of periprosthetic inflammation. During an inflammatory response, IL-6 is produced, which inhibits the generation of T regulatory cells in the periphery and induces the development of TH17 cells from naïve $CD4^+$ cells. In cases of subclinical infection or hematoma organization, the immune system is also activated; therefore, T regulatory cell generation in the periphery is suppressed. In concert with IL-17 and interferon-γ, both responsible for the TH1/TH17–weighted immune response, IL-6 and IL-8 were the most prominent cytokines in the supernatant of the intracapsular T cells. Interestingly, IL-17 was also prominently present in ex vivo capsular tissue, as shown using polymerase chain reaction (Fig. 4, *below*). In most individuals, intracapsular T cells displayed a restricted T cell receptor repertoire when compared with their peripheral blood counterparts. These results strongly argue for an antigen-driven T cell proliferation within the capsular tissue.

CONCLUSIONS

Our results show that not only is capsular fibrosis a simple low-grade, foreign-body reaction but silicone itself, degradation products of silicone, or silicone particles combined with autologous proteins may trigger a specific local immune response via activated TH1/TH17 cells. Furthermore, we observed an inverse correlation between the severity of capsular fibrosis and the numbers of T regulatory cells present in the fibrotic tissue, suggesting that at an early stage, T regulatory cells might delay capsular fibrosis. The presence of an inflammatory stimulus (e.g., IL-6, IL-1β secretion), however, induces a proinflammatory TH17 response, which cannot be suppressed by T regulatory cells in advanced stages of fibrosis development.

Dolores Wolfram, M.D.
Department of Plastic and Reconstructive Surgery
Innsbruck Medical University
Anichstrasse 35
6020 Innsbruck, Austria
dolores.wolfram@i-med.ac.at

ACKNOWLEDGMENTS

The authors thank Prof. Gerhard Pierer (Department of Plastic and Reconstructive Surgery, Medical University Innsbruck) and Dr. Aleksandar Backovic for expert advice and logistic support, and Dr. Dietmar Herndler-Brandstetter, Daniela Niederwieser, and Dr. Karin Jöhrer-Deym for help with the immunoscope and cytokine analyses. They are also grateful to senior plastic surgeon Dr. Barbara Del-Frari, to Prof. Heribert Hussl for providing capsular tissue, to Dr. Jürgen Besemer (Novartis, Vienna, Austria) for providing recombinant human IL-2, and to Rajam Csordas-Iyer for editorial assistance.

REFERENCES

1. Appleton BE, Lee P. The development of systemic-sclerosis (scleroderma) following augmentation mammaplasty. *J Rheumatol.* 1993;20:1052–1054.
2. Brody GS. On the safety of breast implants. *Plast Reconstr Surg.* 1997;100:1314–1321.
3. Brown SL, Pennello G, Berg WA, Soo MS, Middleton MS. Silicone gel breast implant rupture, extracapsular silicone, and health status in a population of women. *J Rheumatol.* 2001;28:996–1003.
4. Henriksen TF, Holmich LR, Fryzek JP, et al. Incidence and severity of short-term complications after breast augmentation: Results from a nationwide breast implant registry. *Ann Plast Surg.* 2003;51:531–539.
5. Alfano C, Mazzocchi M, Scuderi N. Mammary compliance: An objective measurement of capsular contracture. *Aesthetic Plast Surg.* 2004;28:75–79.
6. Fisher JC. The silicone controversy: When will science prevail? *N Engl J Med.* 1992;326:1696–1698.
7. Gerszten PC. A formal risk assessment of silicone breast implants. *Biomaterials* 1999;20:1063–1069.
8. Gylbert L, Asplund O, Jurell G. Capsular contracture after breast reconstruction with silicone-gel and saline-filled implants: A 6-year follow-up. *Plast Reconstr Surg.* 1990;85:373–377.
9. Handel N, Cordray T, Gutierrez J, Jensen JA. A long-term study of outcomes, complications, and patient satisfaction with breast implants. *Plast Reconstr Surg.* 2006;117:757–767.
10. Henriksen TF, Fryzek JP, Holmich LR, et al. Surgical intervention and capsular contracture after breast augmentation: A prospective study of risk factors. *Ann Plast Surg.* 2005;54:343–351.
11. Backovic A, Huang HL, Del Frari B, Piza H, Huber LA, Wick G. Identification and dynamics of proteins adhering to the surface of medical silicones in vivo and in vitro. *J Proteome Res.* 2007;6:376–381.
12. Wolfram D, Rainer C, Niederegger H, Piza H, Wick G. Cellular and molecular composition of fibrous capsules formed around silicone breast implants with special focus on local immune reactions. *J Autoimmun.* 2004;23:81–91; erratum 2005;24:361.
13. Shanklin DR, Smalley DL. The immunopathology of siliconosis: History, clinical presentation, and relation to silicosis and the chemistry of silicon and silicone. *Immunol Res.* 1998;18:125–173.
14. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4(+)CD25(+) T cells. *Clin Immunol.* 2005;115:10–18.
15. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med.* 2001;193:1285–1294.
16. Pfoertner S, Jeron A, Probst-Kepper M, et al. Signatures of human regulatory T cells: An encounter with old friends and new players. *Genome Biol.* 2006;7:R54.
17. Suen JL, Li HT, Jong YJ, Chiang BL, Yen JH. Altered homeostasis of CD4(+) FoxP3(+) regulatory T-cell subpopulations in systemic lupus erythematosus. *Immunology* 2009; 127:196–205.
18. Thornton AM, Shevach EM. CD4(+)CD25(+) immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med.* 1998;188: 287–296.
19. Venigalla RK, Tretter T, Krienke S, et al. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25(high), CD127(-/low) regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum.* 2008;58:2120–2130.
20. Wilczynski JR, Radwan M, Kalinka J. The characterization and role of regulatory T cells in immune reactions. *Front Biosci.* 2008;13:2266–2274.
21. Crome SQ, Wang AY, Levings MK. Translational mini-review series on Th17 cells: Function and regulation of human T helper 17 cells in health and disease. *Clin Exp Immunol.* 2010;159:109–119.
22. Yang XO, Nurieva R, Martinez GJ, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 2008;29:44–56.
23. Yoo ZB, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK, Armitage RJ. Human IL-17: A novel cytokine derived from T cells. *J Immunol.* 1995;155:5483–5486.
24. Awasthi A, Murugaiyan G, Kuchroo VK. Interplay between effector Th17 and regulatory T cells. *J Clin Immunol.* 2008; 28:660–670.
25. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol.* 2009;27:485–517.
26. Erdman SE, Rao VP, Olipitz W, et al. Unifying roles for regulatory T cells and inflammation in cancer. *Int J Cancer* 2010;126:1651–1665.
27. Passos ST, Silver JS, O'Hara AC, Sehy D, Stumhofer JS, Hunter CA. IL-6 promotes NK cell production of IL-17 during toxoplasmosis. *J Immunol.* 2010;184:1776–1783.
28. Baker JL. Augmentation mammaplasty: A comparative analysis (comment). *Plast Reconstr Surg.* 1999;103:1763–1765.
29. Herndler-Brandstetter D, Schwaiger S, Veel E, et al. CD25-expressing CD8(+) T cells are potent memory cells in old age. *J Immunol.* 2005;175:1566–1574.
30. Lo Re S, Dumoutier L, Couillin I, et al. IL-17A-producing gammadelta T and TH17 lymphocytes mediate lung inflammation but not fibrosis in experimental silicosis. *J Immunol.* 2010;184:6367–6377.
31. Simonian PL, Roark CL, Born WK, O'Brien RL, Fontenot AP. Gammadelta T cells and TH17 cytokines in hypersensitivity pneumonitis and lung fibrosis. *Transl Res.* 2009;154:222–227.
32. Kotsianidis I, Nakou E, Bouchliou I, et al. Global impairment of CD4+CD25+Foxp3+regulatory T cells in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med.* 2009;179:1121–1130.
33. Sasaki M, Ikeda H, Sawada S, Sato Y, Nakanuma Y. Naturally-occuring regulatory T cells are increased in inflamed portal

tracts with cholangiopathy in primary biliary cirrhosis. *J Clin Pathol.* 2007;60:1102–1107.
34. O'Connor RA, Taams LS, Anderton SM. Translational mini-review series on Th17 Cells: CD4+T helper cells: Functional plasticity and differential sensitivity to regulatory T cell-mediated regulation. *Clin Exp Immunol.* 2010;159:137–147.
35. Annunziato F, Cosmi L, Santarlasci V, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med.* 2007; 204:1849–1861.
36. Evans HG, Suddason T, Jackson I, Taams LS, Lord GM. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. *Proc Natl Acad Sci USA.* 2007;104:17034–17039.
37. Lohr J, Knoechel B, Wang JJ, Villarino AV, Abbas AK. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J Exp Med.* 2006;203:2785–2791.
38. Stummvoll GH, DiPaolo RJ, Huter EN, et al. Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. *J Immunol.* 2008;181:1908–1916.
39. Bettelli E, Oukka M, Kuchroo VK. TH17 cells in the circle of immunity and autoimmunity. *Nat Immunol.* 2007;8:345–350.
40. Deknuydt F, Bioley G, Valmori D, Ayyoub M. IL-1 beta and IL-2 convert human Treg into T(H)17 cells. *Clin Immunol.* 2009;131:298–307.
41. Koenen HJ, Smeets RL Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25(high)Foxp3(pos) regulatory T cells differentiate into IL-17-producing cells. *Blood* 2008;112:2340–2352.
42. Radhakrishnan S, Cabrera R, Schenk EL, et al. Retraction: Reprogrammed FoxP3(+) T regulatory cells become IL-17(+) antigen-specific autoimmune effectors in vitro and in vivo. *J Immunol.* 2010;184:6556.
43. Tamboto H, Vickery K, Deva AK. Subclinical (biofilm) infection causes capsular contracture in a porcine model following augmentation mammaplasty. *Plast Reconstr Surg.* 2010; 126:835–842.