Human Circulating CD4+CD25highFoxp3+ Regulatory T Cells Kill Autologous CD8+ but Not CD4+ Responder Cells by Fas-Mediated Apoptosis

Laura Strauss, Christoph Bergmann and Theresa L. Whiteside

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Human Circulating CD4+CD25\textsuperscript{high}Foxp3\textsuperscript{+} Regulatory T Cells Kill Autologous CD8\textsuperscript{+} but Not CD4\textsuperscript{+} Responder Cells by Fas-Mediated Apoptosis\textsuperscript{1}

Laura Strauss,* Christoph Bergmann,*‡ and Theresa L. Whiteside\textsuperscript{2*†}

Mechanisms utilized by human regulatory T cells (Treg) for elimination of effector cells may vary. We investigated the possibility that the mechanism of Treg suppression depends on Fas/FasL-mediated apoptosis of responder cells (RC). CD4\textsuperscript{+}CD25\textsuperscript{high}Foxp3\textsuperscript{+} Treg and autologous CD4\textsuperscript{+}CD25\textsuperscript{−} and CD8\textsuperscript{+}CD25\textsuperscript{−} subsets of RC were isolated from blood of 25 cancer patients and 15 normal controls and cocultured in the presence of OKT3 and IL-2 (150 or 1000 IU/ml). Suppression of RC proliferation was measured in CFSE assays. RC and Treg apoptosis was monitored by 7-aminoactinomycin D staining in flow-based cytotoxicity assays. Treg from all subjects expressed CD95\textsuperscript{+}, but only Treg from cancer patients expressed CD95L. These Treg, when activated via TCR plus IL-2, up-regulated CD95 and CD95L expression (p < 0.001) and suppressed CD8\textsuperscript{+} RC proliferation (p < 0.001) by inducing Fas-mediated apoptosis. However, Treg cocultured with CD4\textsuperscript{+} RC suppressed proliferation independently of Fas/FasL. In cocultures, Treg were found to be resistant to apoptosis in the presence of 1000 IU/ml IL-2, but at lower IL-2 concentrations (150 IU/ml) they became susceptible to RC-induced death. Thus, Treg and RC can reciprocally regulate Treg survival, depending on IL-2 concentrations present in cocultures. This divergent IL-2-dependent resistance or sensitivity of Treg and RC to apoptosis is amplified in patients with cancer. *The Journal of Immunology, 2009, 182: 1469–1480.

R egulatory T cells (Treg)\textsuperscript{3} maintain peripheral tolerance by suppression of proliferating effector T cells (1–4). Treg are necessary for the control of immune responses and their depletion in autoimmunity, and conversely, their expansion in cancer is associated with human disease. Regulation of developing immune responses requires that the functional potential and ratios of Treg and effector T cells are strictly controlled. Apoptosis or programmed cell death of responder T cells (RC) imposes a degree of control on immune responses that develops following antigenic stimulation, and the integrity of apoptotic pathways is required for the maintenance of peripheral tolerance.

T cell activation involves a highly coordinated sequence of events. Signaling via TCR or signal 1, costimulation (signal 2), and cytokine support (signal 3) are all needed for mounting an effective immune response and for RC proliferation and differentiation (5). However, although proliferation of activated T cells is necessary, it cannot proceed forever, and Treg are expected to play a key role in the contraction of immune responses. Recently, Treg have been shown to suppress immune responses not only to self epitopes but also to foreign Ags, including transplantation and tumor-associated Ags (6, 7). Furthermore, Treg appear to be able to inhibit multiple stages of T cell activity: proliferation, differentiation, as well as effector function (8). Mechanisms employed by Treg to mediate suppression of T cell functions at these various levels are not yet understood and remain a matter of considerable controversy.

To maintain lymphocyte homeostasis, the coordinated engagement of apoptotic events involving activation-induced cell death (AICD) of T cells or spontaneous T cell death is necessary (9–11). T lymphocytes become vulnerable to apoptosis at critical points of their lifespan, at the time when clonal expansion ends and the contraction phase begins (9). However, it remains unclear how the numbers of human CD4\textsuperscript{+}CD25\textsuperscript{high}Foxp3\textsuperscript{+} T cells are regulated or when the Treg subset expands or how Treg exert suppression in the course of the acute and chronic immune responses. We and others have shown that the frequency and suppressor function of Treg increase in the peripheral circulation of patients with cancer (2, 12–14). Evidence for their depletion in autoimmune diseases is well documented (15–17). These and other observations suggest that the mechanisms involved in control of Treg numbers and function are of critical importance for human health and disease.

Human Treg have been shown to express Fas and FasL (2, 12). Therefore, the use of the Fas/FasL pathway by Treg as one mechanism of suppression is expected based on their phenotypic characteristics (2, 10, 18). We hypothesize that suppression mediated by Treg engages the Fas/FasL pathway, resulting in apoptosis of RC. Also, Treg might become susceptible to Fas-mediated apoptosis during their interaction with RC. However, Treg sensitivity/resistance to this pathway have also been a subject of considerable controversy in the literature (19–21).

In this paper, we use an in vitro system established with circulating CD4\textsuperscript{+} or CD8\textsuperscript{+} T lymphocytes and autologous Treg obtained from peripheral blood of patients with cancer and normal
controls (NC) to investigate whether the mechanism of Treg suppression depends on Fas/FasL-mediated apoptosis of RC. We demonstrate that in Treg/RC cocultures, these cells can reciprocally regulate each other’s survival. Activated human CD4+CD25hi Foxp3+ Treg induce Fas-mediated apoptosis in autologous CD8+ RC, and this mechanism is in part responsible for suppression of RC proliferation. In contrast, CD4+ RC are resistant to Fas-mediated killing by Treg but are able to induce death of Treg in cocultures containing lower concentrations of IL-2. Furthermore, in patients with cancer, these killing mechanisms are amplified, providing a partial explanation for the death of CD8+ in patients with cancer, these killing mechanisms are amplified, cocultures containing lower concentrations of IL-2. Furthermore, killed Treg but are able to induce death of Treg in autologous CD8+ RC. We demonstrate that in Treg/RC cocultures, these cells can reciprocally regulate each other’s survival. Activated human CD4+CD25hi Foxp3+ Treg induce Fas-mediated apoptosis in autologous CD8+ RC, and this mechanism is in part responsible for suppression of RC proliferation. In contrast, CD4+ RC are resistant to Fas-mediated killing by Treg but are able to induce death of Treg in cocultures containing lower concentrations of IL-2. Furthermore, in patients with cancer, these killing mechanisms are amplified, providing a partial explanation for the death of CD8+ effector T cells and a relative resistance of CD4+ T cells to apoptosis observed in cancer (11).

Materials and Methods

Head and neck squamous cell carcinoma (HNSCC) patients and healthy volunteers

Blood samples were obtained from 25 HNSCC patients and 15 age-matched healthy controls (HC). All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. All patients were seen at the Outpatient Otolaryngology Clinic at the University of Pittsburgh Cancer Institute between January 2006 and April 2007. The patient cohort included 17 males and 8 females, with a mean age of 60 years (range, 23–82 years). The NC group included 10 males and 5 females, with a mean age of 60 years. Of patients seen, 22 had a primary disease and 3 had a recurrent disease. At the time of the blood draw, 7 patients were untreated and 18 received various forms of oncologic therapy. The therapy, if given, was terminated from 3 wk to 12 mo before the time of phlebotomy for this study. The age, sex, and clinicopathologic characteristics of the patients are listed in Table I.

<table>
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<tr>
<th>Age (years): mean range</th>
<th>23–82</th>
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<tr>
<td>Sex</td>
<td>Male: 17, Female: 8</td>
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<tr>
<td>Tumor site</td>
<td>Nasal cavity: 2, Oral cavity: 15, Oropharynx: 2, Hypopharynx: 1, Larynx: 4, Not determined: 1</td>
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<td>Tumor differentiation</td>
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<tr>
<td>Nodal status</td>
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<td>Status at blood draw</td>
<td>Primary disease: 22, Recurrent disease: 3</td>
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<tr>
<td>Therapy prior to blood draw</td>
<td>Surgery: 18, Radiotherapy: 11, Radiochemotherapy: 6</td>
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</tbody>
</table>

Table I. Clinicopathologic characteristics of patients with HNSCC who donated PBMC for this study

<table>
<thead>
<tr>
<th>Marker</th>
<th>Normal controls (n = 15)</th>
<th>HNSCC patients (n = 25)</th>
</tr>
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<tr>
<td>% CD4+CD25hiFoxp3+ within the CD4+CD25hi gate</td>
<td>65 ± 20</td>
<td>86 ± 12</td>
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</tbody>
</table>

* The data are mean percentages ± SD of CD4+CD25hiFoxp3 cells within the CD4+CD25hi gate measured by multicolor flow cytometry in the freshly obtained peripheral blood of NC and patients with HNSCC.

Collection of PBMC

Peripheral venous blood (20–30 ml) was drawn into heparinized tubes. The samples were hand carried to the laboratory and immediately centrifuged on Ficoll-Hypaque. PBMC were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

Isolation of T cell subsets from PBMC

CD4+CD25hi T cells from PBMC of HNSCC patients and NC were single cell sorted using the gating strategy previously described by us, in which the threshold for CD25hi cells was established at the mean fluorescence intensity of 120 (12). A MoFlo high-speed cell sorter (Dako) was used for cell separations. The CD4+CD25+ and CD4+CD25hi cell fractions were collected and routinely tested for expression of Foxp3 by flow cytometry and viability in a trypan blue dye exclusion assays. The purity of CD4+CD25hi cells obtained from the patients was usually ~95–98%, while the CD4+CD25hi fractions obtained from NC contained 70–80% CD4+CD25hiFoxp3+ Treg. Sorted cells were immediately used for establishing cocultures.

Antibodies

The following anti-human mAbs (mAb) were used for flow cytometry: anti-CD3-EC, anti-CD4-PC5, anti-CD8-PC5, anti-CD25-FTIC, anti-Foxp3-FTIC (clone PCH101), anti-Fas-FTIC, anti-FasL-PE (NOK-1, 40 and 26 kDa), anti-CD25-PE, and anti-CD152-PE (CTLA-4). Abs and their respective isotypes (purified unlabeled mouse IgG1 and IgG2a), used as negative controls for surface and intracellular staining, were all purchased from Beckman Coulter, except for anti-Foxp3-FTIC and the respective rat IgG2a isotype control (eBioscience). Before use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

Surface and intracellular staining

To determine the frequency of CD4+CD25hi, CD8+CD25hi, CD4+CD25+, and CD8+CD25+ T cells and the expression of Treg markers Foxp3 and CTLA-4, freshly obtained PBMC (at least 2 × 106 cells/tube) were stained with mAbs included in the above-described panel for 15 min at 4°C. Appropriate isotype Ab controls were used in all experiments. Cells were washed and examined by four-color flow cytometry, as previously described (22).

Intracellular staining for Foxp3 or CD152 (CTLA-4) was also performed as previously described (22). Briefly, samples were first incubated with mAbs against surface markers CD4, CD8, CD3, and CD25. After washing, cells were fixed with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature, washed once with PBS containing 0.5% (v/v) BSA and 2 mM EDTA, permeabilized with PBS containing 0.5% BSA and 0.1% (v/v) saponin, and stained with the pre-titrated anti-Foxp3-FTIC for 30 min at room temperature. Cells were further washed twice with PBS containing 0.5% BSA and 0.2% (v/v) saponin, resuspended in FACs flow solution, and analyzed by flow cytometry. Appropriate isotype controls were included for each sample.

Detection of annexin V (AnxV)+ cells

PBMC immediately after their isolation, as well as PBMC ex vivo-activated with OKT3 or IL-2, were analyzed for AnxV binding (% AnxV+) using flow cytometry. Samples were stained with FITC-conjugated AnxV (Molecular Probes) and propidium iodide (Molecular Probes) according to the instructions provided by the manufacturer.

Flow cytometry

Flow cytometry was performed using a FACSscan flow cytometer (Beckman Coulter) equipped with Expo32 software (Beckman Coulter). The
acquisition and analysis gates were restricted to the lymphocyte gate as determined by their characteristic forward (FSC) and side scatter (SSC) properties. FSC and SSC were set in a linear scale. For analysis of all apoptosis-related proteins, at least 10^6 lymphocytes were acquired. Furthermore, analysis gates were restricted to the CD3^-/CD4^-/CD25^- T cell subsets, as appropriate. Cells expressing Treg marker, apoptosis-related proteins, or death molecules were acquired and analyzed in the FL1 or FL2 logarithmic scale using the set gates.

**Flow cytometry-based cytotoxicity assay (FLOCA)**

An in vivo FLOCA was a modification of the assay described by Grossman and colleagues (19). Single-cell sorted, fresh CD4^+CD25^{high} T cell populations were tested for regulatory function by coculture with at least 0.5 x 10^5 CFSE-labeled autologous CD4^-CD25^- or CD8^-CD25^- RC at the Treg/RC ratios of 1:1 or 1:5. The cocultures were incubated for 5 days. CD4^-CD25^- or CD8^-CD25^- T cells separated by SCS were labeled with 1.5 M CFSE (Molecular Probes/Invitrogen) for 10 min at room temperature. The CFSE label was quenched by the addition of an equal volume of FCS (Invitrogen), and then cells were washed extensively with PBS. Treg/RC in cocultures were stimulated with bead-bound or soluble OKT3 (1 g/ml) (American Type Culture Collection) and soluble anti-CD28 Ab in the presence of 150 or 1000 IU of IL-2/ml. Immediately before analysis, cell samples were stained for surface CD4 or CD8 with mAb CD4-ECD or CD8-ECD, respectively, for 15 min at 4°C. After 20 min, samples were resuspended in 500 µl of PBS and immediately analyzed by flow cytometry. In this assay, 7-AAD dye stained dead cells, and live cells were discriminated by their CFSE fluorescence. The percentages of proliferation inhibition and of 7-AAD^+ cells are indicated in respective panels.
incorporation was used as a surrogate marker for late cell death/apoptosis, as it intercalates with DNA in cells that have lost membrane integrity. In many instances, diffuse staining of 7-AAD/H11002 T cells was observed following coculture. This might be a consequence of down-regulation of CD4 or CD8 molecules in activated T cells.

T cell populations were classified as suppressive if they inhibited proliferation of the CD4/H11001 CD25/H11002 or CD8/H11001 CD25/H11002 RC in the coculture assay, and if decreasing the number of CD4/H11001 CD25/H11002 T cells relative to the number of CD4/H11001 CD25/H11002 or CD8/H11001 CD25/H11002 RC in coculture restored proliferation. CD4/H11001 CD25/H11002 T cell populations that satisfied both of these criteria were classified as suppressor T cells. These criteria were applied to all populations so that they could be tested regardless of levels of expansion.

All CFSE data were analyzed using the ModFit software provided by Verity Software House. The percentages of suppression were calculated based on the proliferation index of RC alone compared with the proliferation index of cultures containing RC and Treg. The program determines the percentage of cells within each peak, and the sum of all peaks in the control culture is taken as 100% of proliferation and 0% of suppression.

Transwell assays

To assess whether cell-to-cell contact was necessary for Treg to mediate suppression, polycarbonate 24-well transwell inserts (0.4 μM) (Corning Costar) were used. At least 5 × 10⁴ CFSE-labeled CD4/H11001 CD25/H11002 or CD8/H11001 CD25/H11002 T cells were stimulated with soluble or bead-bound OKT3 and soluble anti-CD28 Ab (each at 1 g/ml) in the presence of 150 or 1000 IU of IL-2/ml in the lower chambers of the plates. Autologous Treg or control cells (CD4/H11001 CD25/H11002 or CD8/H11001 CD25/H11002) were added to the upper chambers at the stimulator/responder ratio of 1:1 or 1:5.

FIGURE 2. Human Treg suppress proliferation but do not mediate apoptosis in CD4⁺ RC. Proliferation and death (% 7-AAD⁻) of CD4⁺ RC alone or CD4⁺ RC in cocultures with Treg isolated from the peripheral blood of a NC and a HNSCC patient were measured in FLOCA. Note that in the coculture with the patient’s cells nearly all CD4⁺ CSFE⁺ Treg are 7-AAD⁺ following incubation with CD4⁺ RC in the presence of OKT3 and 150 IU/ml IL-2. No death is evident in CD4⁺ RC, although their proliferation is suppressed. In contrast, cocultures set up with NC cells show no Treg apoptosis and little suppression of RC proliferation. Results are representative experiments of 10 performed with cells isolated from PBMC of HNSCC patients and 10 performed with cells isolated from PBMC of NC.

<table>
<thead>
<tr>
<th>RC</th>
<th>Proliferation Inhibition (%)</th>
<th>RC⁻</th>
<th>Cell death (%)</th>
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<tbody>
<tr>
<td>CD8⁺ RC</td>
<td></td>
<td></td>
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<tr>
<td>NC (n = 15)</td>
<td>32 ± 19</td>
<td>7.2 ± 0.35</td>
<td>12 ± 10</td>
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<td>HNSCC patients (n = 25)</td>
<td>78 ± 8</td>
<td>56 ± 12</td>
<td>4 ± 2.5</td>
</tr>
<tr>
<td>CD4⁺ RC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC (n = 15)</td>
<td>12 ± 5.5</td>
<td>3.5 ± 2.5</td>
<td>14 ± 4.5</td>
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<tr>
<td>HNSCC patients (n = 25)</td>
<td>80 ± 2.6</td>
<td>3.2 ± 1.7</td>
<td>52 ± 17</td>
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</table>

* Five-day cocultures of Treg with autologous RC were established in the presence of OKT3 and 150 IU/mL rIL-2 as described in Materials and Methods. Treg and RC populations were isolated from the peripheral blood of normal controls or HNSCC patients. Following coculture, the percentage inhibition of RC proliferation and percentages of 7-AAD⁺ RC and Treg were determined by FLOCA. The data are means ± SD.

** RC are either CD8⁺ or CD4⁺, as indicated in the first column.
Analysis of mechanisms used by human CD4\(^{+}\)CD25\(^{high}\) Treg for suppression of CD4\(^{+}\) or CD8\(^{+}\) RC

The FLOCA was modified and used to evaluate the mechanism of suppression by Treg as follows. Expression of FasL on CD4\(^{+}\)CD25\(^{high}\) Treg was blocked with anti-human FasL-neutralizing Ab before the 5-day coculture with autologous CD4\(^{+}\) or CD8\(^{+}\) RC. Briefly, Treg were incubated with neutralizing anti-human FasL-Ab (NOK-1; BioLegend) at 0.5 \(\mu\)l/100 l for 2 h at 37°C. Controls were incubated with isotype-matched control Ab (purified unlabeled mouse IgG1). The optimal concentration of neutralizing Ab was predetermined using titration assays with OKT3/CD28 Ab-activated Jurkat cells, which express high levels of FasL and Fas. The percentage inhibition of FasL obtained with various concentrations of NOK-1 Ab on days 1, 3, and 5 of culture was determined by coincubating the NOK-1-treated Jurkat cells with Fas\(^{-}\) microvesicles (250 ng/ml) isolated from supernatants of a HNSCC cell line (23). After 4 h of incubation, the percentage of AnxV\(^{-}\) cells in coculture was determined.

Susceptibility of human CD4\(^{+}\) and CD8\(^{+}\) T cell subsets to Fas-mediated apoptosis

To determine whether Fas-expressing T cells obtained from cancer patients or NC are sensitive to Fas-mediated apoptosis, we incubated freshly isolated PBMC with anti-Fas Ab (CH-11 Ab). PBMC were treated with 250 ng/ml/\(\times\)10\(^6\) cells of CH-11 Ab for 4 h. We have shown before that at this concentration 90 \(\pm\) 15% of activated Jurkat cells bind AnxV (22). Additionally, PBMC activated with soluble OKT3 (1 \(\mu\)g/ml) and/or IL-2 (150 or 1000 IU/ml) for 48 h were also incubated with CH-11 Ab.

Statistical analysis

Differences between groups were assessed using Wilcoxon test with \(p\) values adjusted by resampling (2000 permutations) and a signed rank test (no adjustment). Values of \(p\) \(\leq\) 0.05 were considered significant.

Results

A phenotypic profile of Treg in the peripheral circulation of NC and patients with HNSCC

Fresh peripheral blood obtained from 25 patients with HNSCC and 15 NC was separated on Ficoll-Hypaque gradients, and PBMC were phenotyped by multiparameter flow cytometry using a previously described panel of markers for Treg (12, 22). The data in Table II show that the mean percentage of CD4\(^{+}\)CD25\(^{high}\)Foxp3\(^{+}\) T cells within the CD4\(^{+}\)CD25\(^{high}\) gate was significantly higher for HNSCC patients than for NC in the peripheral circulation, confirming our earlier results (12). When the patients’ PBMC were single cell sorted based on CD25\(^{high}\) expression (i.e., \(\geq\)120 mean fluorescence intensity) and checked for purity, the Treg fractions contained from 75% to 95% of CD3\(^{+}\)CD25\(^{high}\)Foxp3\(^{+}\) T cells. In
contrast, the purity of Treg isolated by single cell sorting from PBMC of NC was lower, ranging from 60% to 70%. The purity of single cell-sorted CD8\(^{+}\)CD25\(^{+}\) or CD4\(^{+}\)CD25\(^{-}\) T cells ranged from 95% to 98%. All isolated Treg and RC were viable as determined by a trypan blue dye exclusion assay.

**Human CD4\(^{+}\)CD25\(^{hi}\)Foxp3\(^{+}\) Treg suppress proliferation and induce apoptosis in autologous CD8\(^{+}\) RC**

We and others have shown that human CD4\(^{+}\)CD25\(^{hi}\)Foxp3\(^{+}\) Treg obtained from the peripheral blood suppress proliferation of autologous as well as allogeneic CD4\(^{+}\) or CD8\(^{+}\) T cells via direct cell-to-cell contact (12, 24–26). To determine whether CD4\(^{+}\)CD25\(^{hi}\)Foxp3\(^{+}\) Treg isolated from patients with HNSCC or NC induce apoptosis in CD8\(^{+}\) RC, we used FLOCA. As shown in Fig. 1, FLOCA discriminates between CFSE-labeled, 7-AAD\(^{+}\) (dead) RC (left lower panels) and unlabeled, 7-AAD\(^{-}\) (live) Treg (right panels) and simultaneously measures the extent of proliferation inhibition (CFSE dye dilution) in RC populations. In a coculture (RC plus Treg) established with NC cells (Fig. 1A), Treg induced little apoptosis (1.2% of 7-AAD\(^{+}\) RC), and they mediated moderate suppression (32%) of RC proliferation. As indicated in Table III, suppressor function (mean ± SD) for all 15 NC tested was 32 ± 19%, and the percentages of 7-AAD\(^{+}\) cells were 7.2 ± 0.35 and 3.3 ± 2.5 for CD8\(^{+}\) or CD4\(^{+}\) RC, respectively (means ± SD). These results indicate a considerable variability in suppressor function among NC, which is in agreement with our previously reported data (12, 22, 27). Furthermore, a great majority of Treg were 7-AAD\(^{-}\) following cocultures (Fig. 1), which were supplemented with 150 IU/ml rIL-2.

In contrast, CD4\(^{+}\)CD25\(^{hi}\)Foxp3\(^{+}\) Treg obtained from the peripheral blood of patients with HNSCC induced considerably more death in autologous CD8\(^{+}\) RC (up to 80% 7-AAD\(^{+}\) cells) during 5-day cocultures and strongly inhibited RC proliferation (89%) (Fig. 1B and Table III). In control cocultures with autologous CD4\(^{+}\)CD25\(^{-}\) T cells added in place of Treg, there was no suppression. These observations were highly reproducible, and the data for all patients are shown in Table III. In cocultures established in the presence of OKT3 and 150 IU/ml rIL-2, the difference in suppression between Treg in NC vs patients was highly significant with values of p < 0.002 for CD8\(^{+}\) and p < 0.0001 for CD4\(^{+}\) RC (Table III).

**Human CD4\(^{+}\)CD25\(^{hi}\) Treg suppress proliferation but do not mediate apoptosis in autologous CD4\(^{+}\) RC**

When CD4\(^{+}\)CD25\(^{hi}\) Treg isolated from PBMC of HNSCC patients were cocultured with autologous CD4\(^{+}\)CD25\(^{-}\) RC in the presence of OKT3 and 150 IU/ml rIL-2, they suppressed proliferation but did not induce apoptosis in these RC at the Treg/RC ratio of 1:1 (Fig. 2) or 1:5 (data not shown). Treg isolated from NC were only weak suppressors of CD4\(^{+}\) RC proliferation and did not induce apoptosis (data not shown). Surprisingly, CD4\(^{+}\) CFSE\(^{-}\) Treg cells isolated from HNSCC patients and cocultured with autologous CD4\(^{+}\) RC (at Treg/RC ratios of 1:1 or 1:5) became largely 7-AAD\(^{+}\) (97% in Fig. 2, bottom right panel). For cocultures of all HNSCC patients, 7AAD\(^{+}\) Treg were 52 ± 17% vs 14 ± 4.5% for NC (Table III). These data suggested that Treg in cocultures with CD4\(^{+}\) RC mediate suppression of RC proliferation but then become sensitive to apoptosis and are “killed.” Presumably, activated CD4\(^{+}\) RC in the cocultures containing OKT3 and 150 IU/ml rIL-2 are responsible for Treg death.

**The role of IL-2 in death of CD4\(^{+}\)CD25\(^{hi}\) Treg in autologous Treg/CD4\(^{+}\) RC cocultures**

It was unclear why Treg undergo apoptosis following coculture with activated CD4\(^{+}\) RC. However, we have previously
observed that OKT3-activated human CD4⁺CD25<sup>high</sup> Treg become resistant to apoptosis in the presence of high-dose IL-2 (1000 IU/ml), whereas autologous CD4⁺CD25<sup>+</sup> RC are highly sensitive (28). Because it has been reported that human Treg are dependent on exogenous IL-2 for their suppressor activity (29), we suspected that the IL-2 concentration in cocultures played a key role in RC suppression by Treg. To confirm this, we set up cocultures of Treg obtained from patients with HNSCC with OKT3-activated CD4⁺CD25<sup>high</sup> RC in the absence of IL-2 as well as in the presence of low (150 IU/ml) and high (1000 IU/ml) doses of IL-2 (Fig. 3). In a representative coculture stimulated with OKT3 in the presence of 1000 IU/ml IL-2, Treg were resistant to apoptosis (7-AAD<sup>+</sup>), while RC were dying (Fig. 3A, left panels). When cocultures were stimulated with 150 IU/ml IL-2, 7-AAD<sup>+</sup> Treg were numerous, while RC were largely 7-AAD<sup>-</sup> (Fig. 3A, right panels). However, when cultured alone in 150 or 1000 IU/ml IL-2, Treg did not die. Similar results were obtained with cocultures established with RC and Treg obtained from five different HNSCC patients (Fig. 3B). In the absence of IL-2 in the cocultures, variable proportions of Treg and RC were 7-AAD<sup>+</sup> on day 5, possibly due to deprivation-induced cell death (Fig. 3B).

As shown in Fig. 3C, Treg cultured in the presence of 1000 IU/ml IL-2 mediated strong suppression of RC proliferation, which was significantly greater than that mediated by Treg activated by 150 IU/ml IL-2 (p < 0.04) or Treg in cocultures with no IL-2 (p < 0.001).

Table IV. Fas and FasL expression on CD4⁺<sup>+</sup> or CD8⁺<sup>+</sup> RC isolated from NC or patients with HNSCC prior to and after culture with OKT3 and IL-2<sup>a</sup>

<table>
<thead>
<tr>
<th>RC</th>
<th>Prior to culture</th>
<th>After culture</th>
<th>p Value</th>
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<tr>
<td>Fas (CD95)</td>
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<td></td>
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<tr>
<td>CD8⁺CD25⁺ NC</td>
<td>32 ± 10</td>
<td>65 ± 13</td>
<td>&lt;0.5</td>
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<td>CD4⁺CD25⁺ HNSCC</td>
<td>45 ± 10.5</td>
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<td>&lt;0.5</td>
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<td>CD4⁺CD25⁺ NC</td>
<td>43 ± 11.2</td>
<td>75 ± 6</td>
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<td>FasL (CD95L)</td>
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<td>CD8⁺CD25⁺ HNSCC</td>
<td>2.1 ± 0.85</td>
<td>6.7 ± 3</td>
<td>&lt;0.5</td>
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<td>CD8⁺CD25⁺ NC</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>CD4⁺CD25⁺ HNSCC</td>
<td>0 ± 3.2</td>
<td>3 ± 1</td>
<td>&lt;0.5</td>
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<sup>a</sup> Isolated Treg or RC were obtained from NC (n = 15) or HNSCC patients (n = 25) and studied fresh or after 48 h of culture in the presence of OKT3 and IL-2 as described in Materials and Methods. The data are mean percentages of positive cells ± SD. The p values refer to differences between fresh vs cultured T cell subsets.
We established comparable cocultures in the presence of the high and low doses of IL-2 with Treg and RC obtained from NC. However, the results were less informative, as percentages of dying cells and proliferation suppression were considerably lower relative to the values obtained with the cells isolated from HNSCC patients (see, e.g., Table III).

Suppression and apoptosis in Treg/RC cocultures is cell contact-dependent

To evaluate whether apoptosis of RC in the presence of IL-2 requires cell-to-cell contact between activated Treg and RC, we established the autologous Treg plus CD4/H11001 RC or Treg plus CD8/H11001 RC cocultures at the Treg/RC ratios of 1:1 and 1:5 using transwell inserts (Tw). In half of the assays, Treg and RC were separated by a membrane eliminating cell-to-cell contact. RC were stimulated with OKT3 in the absence or presence of IL-2 (150 or 1000 IU/ml). With Tw, suppression of CD8/H11001 RC proliferation mediated by CD4/H11001CD25highFoxp3/H11001 Treg isolated from PBMC of three HNSCC patients was nearly completely inhibited at all Treg/RC ratios in cocultures containing IL-2, regardless of its concentration (Fig. 4A, lower panels). Concomitantly, apoptosis of RC in Treg/H11001/CD8/H11001 RC cocultures was significantly, albeit not completely, inhibited in the presence of transwell membranes (Fig. 4A, upper panels). In these cocultures, Treg were not 7-AADD+, except when IL-2 was absent, as noted above.

When Treg/CD4/H11001 RC cocultures were established and activated with lower or higher doses of IL-2, a complete inhibition of CD4/H11001 RC proliferation in the presence of Tw was again observed (Fig. 4B, lower panels). At the lower IL-2 concentration, most Treg became 7-AADD− in cocultures with no Tw, while in the presence of Tw, death of Treg was abolished (Fig. 4B, upper panels), suggesting that cell-to-cell contact is essential for Treg demise. In cocultures set up in the higher IL-2 concentration, Treg remained 7-AADD− (no death) with or without Tw, while CD4/H11001 RC were 7-AADD− with or without Tw. This result suggests that death of CD4/H11001 RC is independent on cell-to-cell contact. When cocultures contained no IL-2, death of Treg was significantly greater than for that of CD4/H11001 RC in the absence of Tw, but not in its presence (Fig. 4B). Taken together, these results demonstrate that Treg as well as RC can induce death and deplete their “partner” cells in cocultures activated with OKT3 and IL-2. Proliferation, suppression, and death of the CD8/H11001 partner cell requires direct cell-to-cell contact, but death of the CD4/H11001 partner might depend in part on soluble factors. The fate of Treg and CD8/H11001 or CD4/H11001 RC may be decided by the concentration of IL-2 in the coculture, with low IL-2 concentrations favoring Treg death. Thus, Treg of patients with cancer are highly sensitive to contact-dependent...
apoptosis at 150 IU/ml IL-2, while at 1000 IU/ml IL-2, only CD4+ RC and not Treg undergo apoptosis.

Expression of Fas/FasL on human CD4+CD25high Foxp3+ Treg and autologous CD8+ and CD4+ RC

Our results indicated that apoptosis of RC mediated by human CD4+CD25highFoxp3+ Treg required direct cell-to-cell contact and was regulated by signaling pathways induced by T cell activation with OKT3 and IL-2. It has been well documented that IL-2 mediates AICD via the Fas/FasL pathway (30, 31). We therefore looked for Fas and FasL expression on Treg and autologous CD8+ and CD4+ RC by using flow cytometry immediately after their isolation from blood samples and after their ex vivo activation (for 48 h) with OKT3 and 150 IU/ml or 1000 IU/ml IL-2. Samples from HNSCC patients and NC were analyzed using the anti-FasL Ab NOK-1, which reacts with the membrane-bound 40-, 44-, and 46-kDa isoforms of FasL but also recognizes the cleaved and soluble 26-kDa isoforms of FasL.

As we previously reported (32), CD8+CD25− and CD4+CD25− RC isolated from peripheral blood in NC or cancer patients expressed low to intermediate levels of Fas, but no FasL (Fig. 5A and Table IV). After in vitro stimulation with OKT3 and IL-2, Fas was up-regulated on CD4+ and CD8+ RC in NC and cancer patients. However, FasL expression was only slightly increased on the cell surface of in vitro-activated human CD4+ and CD8+ RC (Fig. 5A and Table IV).

Fas was also highly expressed on fresh as well as OKT3- and IL-2-activated CD4+CD25high Treg obtained from NC and HNSCC patients (Fig. 5B). Importantly, expression of Fas and FasL by the CD4+CD25high Treg in patients with HNSCC was significantly greater (p < 0.03 and p ≤ 0.001, respectively) relative to that in NC (Fig. 5C). FasL was only weakly expressed on a subset of fresh Treg obtained from PBMC of NC or HNSCC patients (Fig. 5C). When we activated Treg of NC with OKT3 and IL-2, FasL expression was not up-regulated (Fig. 5B). Surprisingly, however, in vitro-activated CD4+CD25high Treg cells from HNSCC patients expressed...
high levels of FasL on the cell surface (Fig. 5, B and C). These results suggest that in patients with cancer either the cleavage or endocytosis of FasL from the cell surface of activated Treg is avoided or delayed, converting these Treg into potent inducers of apoptosis mediated via the Fas/FasL pathway.

CD4+CD25high Treg from HNSCC patients express FasL but are resistant to Fas-mediated apoptosis

Because activated Treg as well as activated RC expressed high levels of Fas (CD95), it was surmised that these cell subsets might be susceptible to Fas/FasL-mediated apoptosis. Therefore, we analyzed the sensitivity of fresh and OKT3- and IL-2 (150 IU/ml)-activated CD4+CD25high Treg and CD4+ or CD8+ RC to Fas-mediated apoptosis. We incubated freshly isolated and activated T cell subsets with CH-11-Ab (or isotype control Ab) for 4 h and measured the percentage of Annexin V+ cells (Table V). Fresh CD4+CD25− and CD8+CD25− RC subsets obtained from NC were highly sensitive to CH-11 Ab (45 ± 15% and 85 ± 17% Annexin V+ cells, respectively). Susceptibility of CD4+ or CD8+ RC to CH-11 Ab was increased after in vitro stimulation with OKT3 and IL-2 (90 ± 8.5% Annexin V+ cells in Table V). Counterintuitively, however, fresh CD4+CD25high Treg isolated from patients with HNSCC and expressing surface FasL (as shown in Fig. 5) were found to be resistant to CH-11 Ab-induced apoptosis (no Annexin V+ cells in Fig. 6A). Following activation with OKT3 and 150 IU/ml IL-2, CD4+CD25high Treg obtained from NC or HNSCC patients remained resistant to CH-11 Ab-induced apoptosis (Fig. 6B).

Collectively, these results suggest that 1) while OKT3- and IL-2-activated CD4+CD25high Treg become resistant to Fas-mediated apoptosis, CD4+ RC remain highly sensitive; and 2) freshly isolated CD4+CD25high Treg in patients with HNSCC are highly active T cells that are protected from, or are resistant to, apoptosis and remain resistant following ex vivo activation.

Treg can “kill” autologous CD8+CD25− RC but not activated CD4+CD25− RC via the Fas/FasL pathway

As FasL and Fas were coexpressed on CH-11-resistant CD4+CD25high Treg, and Fas was present on CH-11 Ab-sensitive CD8+RC and CD4+RC, we asked whether Treg could use the Fas/FasL pathway to induce apoptosis in autologous CD8+ or CD4+RC, and whether Fas/FasL-mediated apoptosis might be in part responsible for suppression of RC proliferation in Treg/RC cocultures. Therefore, Treg obtained from PBMC of HNSCC patients or NC were coincubated with CFSE-labeled autologous CD8+ or CD4+RC at the Treg/RC ratios of 1:1 or 1:5. As before, RC were stimulated with OKT3 and 150 IU/ml IL-2. Half of the Treg samples were blocked with antagonistic anti-FasL Ab NOK-1 or an isotype control Ab before coculture, as described in Materials and Methods. Suppression of proliferation and apoptosis (7-AAD+) cells of the CFSE− Treg as well as CFSE-labeled RC population were analyzed by FLOCA. As shown in Fig. 7A, Treg isolated from a HNSCC patient suppressed proliferation of autologous CD8+RC, and CD8+RC were sensitive to apoptosis. Treg obtained from patients with HNSCC suppressed proliferation of autologous CD8+RC, and CD8+RC were 7-AAD− (48 ± 18% for all cocultures tested) at the S/RC ratio of 1:1. As expected, Treg from NC were only weakly suppressive and induced little or no apoptosis in autologous CD8+RC (data not shown). When Treg obtained from HNSCC patients were pretreated with FasL-neutralizing Ab and then coincubated with autologous CD8+RC, no 7-AAD+ were present in the RC fraction and suppression of RC proliferation was partially relieved (65 ± 12% for all cocultures tested and 62% of suppression in Fig. 7A). Isotype control Ab did not induce these effects (data not shown). These data suggest that suppression of CD8+RC by Treg is in large part mediated by Fas/FasL-induced apoptosis, although other mechanisms of suppression may also be present.

In contrast to Treg/CD8+RC cocultures, the incubation of Treg with antagonistic FasL-Ab (or isotype control Ab) before coculture with CD4+RC did not inhibit suppression of their proliferation (Fig. 7B) and had no effect on RC cell death, which remained minimal. Thus, suppression of proliferation of CD4+RC by Treg was not mediated via the Fas/FasL pathway (Fig. 7B). Surprisingly, however, Treg in Treg/CD4+RC cocultures became 7-AAD− after coincubation with CD4+CD25+RC (75 ± 15%) (Fig. 7B). The addition of antagonistic FasL Ab did not substantially reduce the number of 7-AAD+ Treg in these cocultures (Fig. 7B). These results suggest that the Fas/FasL pathway is not involved in suppression of CD4+RC by Treg and that activated Treg are not depleted via a “suicide” mediated by the Fas/FasL pathway.

Discussion

Peripheral tolerance mediated by CD4+CD25highFoxp3+nTreg appears to represent a key mechanism for maintaining an immunologic balance and protecting the host from undesirable autoimmune reactivity (33–35). The paucity of nTreg in autoimmune diseases and, conversely, their excess in patients with cancer suggest that Treg imbalance is associated with disease development. Treg abnormalities have been reported in autoimmune diseases, chronic infections, parasitic infestation, and cancer (1, 2, 36–39). Given the impact of dysregulated Treg function in many human diseases, it is important to better understand Treg biology to be able to devise therapies able to effectively restore or equilibrate the immune balance. Therefore, the knowledge of molecular pathways that control and drive Treg activity is necessary, and the understanding of mechanisms Treg use for interactions with other subsets of lymphocytes is a key requirement for the development of corrective therapies in the future.

Mechanisms reported to be responsible for suppressor functions of Treg vary. It appears that Treg can engage many different mechanisms, perhaps depending on the environment in which they operate. Aside from the mechanisms requiring direct physical contact between cells such as receptor-ligand interactions involving, for example, CD95 (20, 21, 40), ICOS (41), or CD46 (42), as well as contact-mediated suppression by granzyme B/perforin produced by Treg (19, 43, 44), a variety of soluble factors mediating RC death have been described. Prominent among these factors are immunoinhibitory cytokines IL-10 and TGF-β (2, 12, 45) and adenosine likely derived from an enzymatic cleavage of ATP by Treg-associated ectonucleoside hydrolases (46, 47). Additionally, cytokine deprivation induced by Treg has been recently proposed as a mechanism inhibiting effector T cell responses (48). Most of the above-listed mechanisms have been identified in murine systems, and there is a paucity of information regarding human Treg, especially in patients with cancer.

In this study, we have examined mechanisms of suppression mediated by human Treg during interactions with RC in an ex vivo model system consisting of cocultures of single cell-sorted CD4+CD25highFoxp3+n and CD4+CD25− or CD8+CD25− RC populations. It was possible to differentiate between CFSE-labeled RC and unlabeled Treg in these cocultures by using the FLOCA assay. Pretreatment of these interacting cell populations with mAbs or preventing their cell-to-cell contact by using transwell inserts enabled us to determine, in part, the mechanisms responsible for suppression of RC proliferation and cell survival or death. Sorted CD4+CD25high Treg used in these experiments were derived either from peripheral blood of NC or HNSCC patients and were positive for Foxp3 and CTLA-4 expression. Treg isolated...
from the blood of patients with cancer were previously shown to mediate high levels of suppression, while Treg isolated from PBMC of NC generally had lower levels of suppressor activity in our cocultures (12, 27). The low levels of suppression mediated by isolated CD4\(^+\)CD25\(^{hi}\) Treg of NC we consistently see are likely due to the presence of IL-2 in the cocultures. Other investigators have reported high levels of suppression in cocultures of Treg and RC in the absence of exogenous IL-2 (e.g., see Ref. 49) probably because the competition for cytokines is the primary mode of suppression in this instance. Thus, Treg consume IL-2, depriving RC of cytokines necessary for proliferation, as also suggested by Pandiyan et al. (48). Furthermore, in “traditional” suppressor assays, most investigators do not measure cell death, but only RC proliferation. In such assays, it would not be possible to discriminate inhibition of proliferation due to RC death mediated by IL-2 starvation from that mediated by the Fas/FasL engagement or the granzyme/perforin pathway.

Treg obtained from the peripheral circulation of cancer patients mediate strong suppression of RC proliferation (2, 10, 27). These Treg are activated as evidenced by high levels of Fas and FasL presentation on the cell surface. We now show that the “killing” potential of Treg is also higher in cancer patients than in NC, and that human CD4\(^+\)CD25\(^{hi}\) Treg arrest proliferation of CD4\(^+\) and CD8\(^+\) RC by distinct mechanisms involving cell-to-cell contact. While CD8\(^+\)CD25\(^-\) RC proliferation is suppressed by Fas/FasL-mediated apoptosis, suppression of CD4\(^+\)CD25\(^{hi}\) RC by Treg is Fas-independent and involves cell death mediated by granzyme B/perforin, as recently described by us (L. Strauss, C. Bergmann, H. Rabinowich, and T. L. Whiteside, submitted for publication) and others (19, 20, 50). In cancer patients, both the frequency and function of Treg are elevated relative to those in NC (12–14). The level of Treg activation is amplified in patients with cancer, whether due to an Ag excess and/or up-regulated cytokine networks. The state of “chronic inflammation” accompanying tumor progression in patients with active disease or that due to tissue distinction after oncologic therapies in patients with no evident disease probably favors rapid and effective Treg expansion as well as a high level of suppressor activity.

The most striking observation we made is that both Treg and RC can reciprocally regulate T cell survival in cocultures, and that Treg can be induced to die in cocultures with activated RC. Furthermore, Treg sensitivity or resistance to RC-derived death signals appears to be dependent on the IL-2 concentration in cocultures. Our results extend to the purified human Treg and RC subsets somewhat similar results reported by Banz et al. in mice (40). As it has been documented that human nTreg express CD25 and that Thy1 express CD132 (IL-2R\(\gamma\)) (45), it is not surprising that exogenous IL-2 plays a key role in their functions (29) and that low vs high IL-2 concentrations might exert differential effects on Treg activities. It is possible that the concentration of 1000 IU/ml IL-2 we used ex vivo is not physiological. However, we and others (e.g., see Ref. 48) have selected this concentration to determine the threshold for Treg survival in Treg-RC cocultures. As Treg are known to avidly consume IL-2, we considered it necessary to use high IL-2 concentrations for ex vivo assays. It is, however, plausible that in the tissue or pericellular microenvironment at the peak of immune responses, levels of IL-2 might be quite substantial. Also, when IL-2 is administered therapeutically in patients with cancer, its levels are high, with a reported concomitant increase in Treg frequency and suppressor activity (51). Thus, our in vitro data could be of biologic if not “physiologic” importance.

In addition to IL-2R, Treg as well as RC express Fas and up-regulate Fas and FasL expression on the cell surface upon activation by OKT3 and IL-2. Thus, activated Treg and RC can potentially eliminate one another by utilizing the Fas/FasL pathway. However, in the presence of high IL-2 concentrations, Treg acquire resistance to Fas-mediated apoptosis and remain viable despite expression of Fas and FasL on their cell surface. In contrast, CD8\(^+\) RC remain sensitive to Fas-mediated apoptosis and are readily eliminated by activated FasL\(^+\) Treg. Interestingly, CD4\(^+\) RC are also resistant to Fas-mediated apoptosis, and suppression of their proliferation by Treg is attributable to different cell contact-dependent mechanisms and/or cytokines, as previously reported by us (2, 45). In the presence of low IL-2 concentrations, Treg are sensitive to death signals induced by CD4\(^+\) RC. In aggregate, these cellular interactions in the coculture system suggest that both Treg and RC might contribute to the control of immune responses but perhaps at different points in their evolution. Thus, at the peak of the response, when T cell proliferation and IL-2 production are high, Treg are resistant to apoptosis and exercise their suppressive function, inducing inhibition of RC proliferation and RC death. As the immune response enters a contraction phase, due in part to Treg-mediated suppression, IL-2 production is decreased, and Treg become sensitive to apoptosis mediated by surviving, activated, and FasL\(^+\) RC. The ability of T cells to reciprocally induce killing of the partner that expresses the correct receptor profile to down-regulate immune response is well documented in the literature (30). Also, the involvement of the Fas/FasL system in this process has been described (18, 20, 21). The use of this mechanism by interacting activated Treg and RC, as documented in our ex vivo experiments, is an excellent example of how immune inhibitory and stimulatory signals are regulated in the specific microenvironment. It is possible that the reciprocal killing contributes to regulating of Treg homeostasis in vivo in health and disease. An excess or paucity of Treg in a given microenvironment might be regulated by T cells themselves, utilizing Fas-dependent or Fas-independent mechanisms to reduce suppression.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


1480 ACTIVATED REGULATORY T CELLS ARE RESISTANT TO APOPTOSIS