Identification of Human Regulatory T Cells in the Setting of T-Cell Activation and Anti–CTLA-4 Immunotherapy on the Basis of Expression of Latency-Associated Peptide

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Effectors and regulatory T cells (Treg) share multiple markers that make it difficult to discern differences in these populations in humans. The transcription factor FoxP3 has been shown to identify Tregs. However, the detection of FoxP3 requires cell permeabilization, thereby preventing isolation of viable Tregs. Subsequently, the extracellular marker CD127 was established for the identification of Tregs. However, these studies were not conducted in the setting of immunotherapy. Here, we conducted studies to analyze CD127 and FoxP3 expression on T cells before and after in vitro activation as well as in the setting of patients treated with antibodies directed against cytotoxic T-lymphocyte antigen-4 (CTLA-4). We show that latency-associated peptide (LAP), as opposed to CD127, was capable of identifying Tregs after in vitro activation as well as after treatment with anti–CTLA-4. Therefore, we propose that LAP should be used as a marker of Tregs for immune monitoring studies in patients treated with active immunotherapy such as anti–CTLA-4.

SIGNIFICANCE: Tregs play an important role in human diseases, including cancer and autoimmunity; however, it has been difficult to study these cells because of a lack of an appropriate marker. Here, we propose LAP as a marker that can be used to identify Tregs in patients treated with immunotherapy, thereby permitting isolation of these cells for functional studies and for ex vivo expansion. Cancer Discovery; 2(2); 122-30. © 2011 AACR.
Further investigations into Treg cell biology led to the identification of the X-chromosome-encoded gene FoxP3. A loss-of-function mutation in the FoxP3 gene leads to an early-onset lymphoproliferative immune-mediated disease that is fatal (5). In subsequent studies, the transcription factor FoxP3 was found to be stably expressed in Tregs and was required for Treg cell differentiation and immunosuppressive function (6, 7). Together, these studies established a central role for FoxP3 in defining the Treg cell lineage.

Given that Foxp3 is an intracellular molecule, it requires fixation and permeabilization of cells to allow for detection. Therefore, although Foxp3 remains the best and most specific marker of Tregs thus far, it cannot be used to isolate viable Treg populations for functional studies or ex vivo expansion. Furthermore, although stable high expression of Foxp3 is restricted to Treg cells in both mice and humans, it has been shown in humans (but not in mice) that Foxp3 expression can be induced after the stimulation of conventional T cells (8, 9), thereby making it difficult to distinguish Tregs from activated effector T cells (Teff) in patients who are treated with active immunotherapy agents.

As a result of the limitations that arise with the use of Foxp3 as a marker of Tregs, investigators have attempted to identify cell-surface markers that correlate with Foxp3 expression. Different groups have reported that surface expression of CD127, the α-chain of the interleukin-7 receptor, in combination with CD25 can distinguish between human regulatory and conventional CD4 T cells (10, 11). However, these studies were not conducted on samples from patients treated with active immunotherapy agents.

Anti–CTLA-4 therapy (ipilimumab; Bristol-Myers Squibb) was approved by the U.S. Food and Drug Administration (FDA) in March 2011 as a standard-of-care treatment for patients with metastatic melanoma after it was shown to improve survival of treated patients in a randomized phase III clinical trial (12). Because anti–CTLA-4 targets a T-cell-specific molecule, as opposed to a tumor cell-specific molecule, it is being investigated as a potential therapeutic agent in multiple types of malignancy (13). Anti–CTLA-4 targets a T-cell molecule known as cytotoxic T-lymphocyte antigen-4 (CTLA-4). CTLA-4 functions to limit T-cell responses (14). In the setting of T-cell activation, which occurs as a result of engagement of T-cell receptor and CD28 costimulation, intracellular signals lead to trafficking of CTLA-4 to the immunologic synapse, whereby CTLA-4 outcompetes CD28 for ligand binding, thus acting as an intrinsic “off” switch to restrict T-cell activity. Anti–CTLA-4 acts to remove this “off” switch, thus allowing for enhanced Teff function and antitumor responses (15). CTLA-4 is also expressed by Tregs (16); however, the impact of anti–CTLA-4 on human Tregs has been difficult to assess because of the fact that both Teff and Tregs express markers that can be affected by T-cell activation.

In an attempt to identify specific extracellular markers of Tregs in the setting of T-cell activation, we conducted flow cytometric studies with CD25, FoxP3, and CD127 on in vitro-activated human peripheral blood mononuclear cells (PBMC) and PBMCs derived from patients treated with anti–CTLA-4 immunotherapy. Here, we report that in the setting of in vitro activation, CD4+CD25+CD127low cells failed to represent Tregs but, a different extracellular marker, latency-associated peptide (LAP), could be used to identify Tregs. LAP was previously shown to be associated with TGF-β, which is expressed on Tregs (17–19). Furthermore, our data indicate that LAP, as opposed to CD127, is a better marker for identification of Tregs after patients have been treated with anti–CTLA-4. In the setting of T-cell activation, LAP permits accurate identification and isolation of Tregs for functional studies.

RESULTS

CD4+CD25+CD127low Cells Correlate with Tregs in the Absence of In Vitro Activation

Because staining for FoxP3 requires cellular permeabilization, which leads to cell death, thereby prohibiting isolation of FoxP3+ T cells for functional studies, many investigators rely on CD127low staining as a surrogate marker for FoxP3+ T cells (10, 11). In our studies, we found that approximately 6% of unactivated human CD4 T cells from normal healthy donors were CD25+FoxP3+ (Fig. 1A, representative individual and Fig. 1B, summary data of 8 healthy donors). Similarly, approximately 7% of unactivated human CD4 T cells from healthy donors were CD25+CD127low (Fig. 1C, left panel), with a predominant proportion (~78%) demonstrating Foxp3 expression, consistent with a Treg phenotype (Fig. 1C, right panel). Summary data from 8 healthy donors are also shown (Fig. 1C, lower panels).

To determine whether the CD4+CD25+CD127low T cells from unactivated samples were capable of functioning as Tregs, we conducted in vitro suppression assays. We isolated CD4+CD25+CD127low T cells from unactivated human PBMCs and found that these cells were capable of suppressing proliferation of autologous CD4+CD25− T cells (Fig. 1D). Increasing numbers of CD4+CD25+CD127low suppressor (S) cells led to decreased proliferation of responder (R) cells with significant suppression (P < 0.01) at a R:S ratio of 1:1, which was consistent with the idea that the CD4+CD25+CD127low cells functioned as Tregs.

CD4+CD25+CD127low Cells Do Not Correlate with Tregs in the Presence of In Vitro Activation

Although there was an observed correlation between CD4 T cells that were FoxP3+ and CD127low in unactivated human PBMCs, this correlation was not observed in the setting of activated human PBMCs. Upon in vitro activation with anti-CD3 and anti-CD28, we observed an approximately 2-fold increase in the frequency of CD4+CD25+FoxP3+ cells (Fig. 2A as compared with Fig. 1A), which is consistent with previously published results indicating an increase in Foxp3 expression upon T-cell activation (8, 9). Summary data from 8 healthy donors are shown in Fig. 2B. Upon T-cell activation, there was also an increase in the frequency of CD4+CD25+CD127low cells (Fig. 2C, right panel) but, interestingly, approximately 67% of these cells were Foxp3+ and approximately 33% of these cells were Foxp3− (Fig. 2C, left panel), which was a marked difference when compared with Foxp3 expression in these
Figure 1. Frequency of CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁺CD127low T cells in unactivated PBMCs from healthy donors. Frequency of FoxP3 expression in CD4⁺CD25⁺ T cells from one representative individual (A) and summary data from 8 healthy donors (B). Frequency of CD127low cells in CD4⁺CD25⁺ T cells from one representative individual (C, right upper panel) with summary data from 8 healthy donors (C, right lower panel) and frequency of FoxP3 T cells in the CD4⁺CD25⁺CD127low population from one representative individual (C, left upper panel) with summary data from 8 healthy donors (C, left lower panel). Representative thymidine incorporation assay, which measured cell proliferation in the presence of media only, CD4⁺CD25⁺ responder (R) cells plus CD4⁺CD25⁺CD127low suppressor (S) cells at R:S ratios of 0:1, 1:0, 1:0.1, 1:0.5, and 1:1 (D). Each experiment was performed in triplicate, and suppression assays were repeated with a minimum of 3 different donor samples. Bars represent mean ± SEM.

cells from unactivated samples (Fig. 1C). Summary data from 8 healthy donors are also shown (Fig. 2C, lower panels). In addition, CD4⁺CD25⁺CD127low T cells that were isolated from activated human PBMCs did not function as Tregs because they were unable to suppress proliferation of autologous CD4⁺CD25⁻ T cells (Fig. 2D). Therefore, CD4⁺CD25⁺CD127low cells correlate with a functional Treg population in the setting of unactivated human T cells but, in the setting of in vitro activation, CD4⁺CD25⁺CD127low cells do not correlate with a functional Treg population.
LAP Identifies Human Tregs after Anti–CTLA-4 Therapy

**Figure 2.** Frequency of CD4+CD25+FoxP3+ and CD4+CD25+CD127low T cells after in vitro activation of human PBMCs. Frequency of FoxP3 expression in CD4+CD25+ T cells from one representative individual (A) and summary data from 8 healthy donors (B). Frequency of CD127+ cells in CD4+CD25+ T cells from one representative individual (C, right upper panel) with summary data from 8 healthy donors (C, right lower panel) and frequency of FoxP3+ T cells in the CD4+CD25+CD127low population from one representative individual (C, left upper panel) with summary data from 8 healthy donors (C, left lower panel). Representative thymidine incorporation assay, which measured cell proliferation in the presence of media only, CD4+CD25+ responder (R) cells plus CD4+CD25+CD127low cells at R:S ratios of 0:1, 1:0, 1:0.1, 1:0.5, and 1:1 (D). Each experiment was performed in triplicate and suppression assays were repeated with a minimum of 3 different donor samples. Bars represent mean ± SEM.

**CD4+CD25+ LAP+ Cells Correlate with Tregs in the Presence of In Vitro Activation**

To determine whether LAP could act as a surrogate marker for Tregs, we analyzed LAP and FoxP3 expression in unactivated and activated human PBMCs. We found that the frequency of CD4+CD25+ LAP+ cells was low (~0.3%) in unactivated samples (Fig. 3A, representative individual and Fig. 3B, summary data from 8 healthy donors). However, upon in vitro activation of PBMCs, the frequency of CD4+CD25+ LAP+ cells increased to approximately 12% (Fig. 3C, right panel), with approximately 82.5% of these cells expressing FoxP3 (Fig. 3C, left panel). Summary data from 8 healthy donors are also shown (Fig. 3C, lower panels). Similarly, in activated CD4 T cells, FoxP3+ T cells are predominantly LAP+ (Fig. 3D, left and right panels). Summary data of 8 healthy donors for frequency of CD4+CD25+FoxP3+ cells (Fig. 2B) and LAP expression in CD4+CD25+FoxP3+ cells (Fig. 3D, lower panel) after in vitro activation are also shown. In addition, CD4+CD25+ LAP+ T cells from activated human PBMCs functioned as Tregs that were capable of significantly suppressing in vitro proliferation of
Figure 3. Frequency of CD4⁺CD25⁺LAP⁺ T cells before and after in vitro activation of human PBMCs. Frequency of LAP expression in CD4⁺CD25⁺ T cells from unactivated human PBMCs in one representative individual (A) and summary data from 8 healthy donors (B). Frequency of LAP expression in CD4⁺CD25⁺ T cells after in vitro activation of human PBMCs in one representative individual (C, right upper panel) with summary data from 8 healthy donors (C, right lower panel) and frequency of FoxP3⁺ T cells in the CD4⁺CD25⁺ LAP⁺ population from one representative individual (C, left upper panel) with summary data from 8 healthy donors (C, left lower panel). Frequency of FoxP3 expression in CD4⁺CD25⁺ T cells after in vitro activation of human PBMCs in one representative individual (D, right upper panel) and frequency of LAP⁺ T cells in the CD4⁺CD25⁺FoxP3⁺ population from one representative individual (D, left upper panel) with summary data from 8 healthy donors (D, left lower panel). Representative thymidine incorporation assay, which measured cell proliferation in the presence of media only, CD4⁺CD25⁻ responder (R) cells plus CD4⁺CD25⁺ LAP⁺ suppressor (S) cells at R:S ratios of 0:1, 1:0, 1:0.1, 1:0.5, and 1:1 (E). Representative CFSE assay with CD4⁺CD25⁻ responder cells alone (F, right panel) and CD4⁺CD25⁻ responder cells plus CD4⁺CD25⁺ LAP⁺ cells at R:S ratio of 1:1 (F, left panel). Each experiment was performed in triplicate, and suppression assays were repeated with a minimum of 3 different donor samples. Bars represent mean ± SEM.
CD4+CD25+ T cells (P < 0.01), as observed by both thymidine incorporation (Fig. 3E) and in carboxyfluorescein diacetate succinimydyl ester assays (Fig. 3F).

CD4+CD25+LAP+ Cells, as Compared to CD4+CD25+CD127low Cells, Correlate with Tregs in the Setting of Anti–CTLA-4 Therapy

We previously reported on a clinical trial whereby 12 patients with bladder cancer were treated with anti–CTLA-4 (ipilimumab) in the presurgical setting (20–22). We and others have reported changes in the frequency of FoxP3+ T cells after treatment with anti–CTLA-4 (21, 23). However, in humans, changes in FoxP3 expression may represent T-cell activation rather than reflect the frequency of Tregs. Here, we assessed FoxP3, CD127, and LAP expression in pre- and posttherapy samples from patients treated with anti–CTLA-4. In the peripheral blood of 12 patients, we observed that 6 patients had an increase in the frequency of CD4+CD25+FoxP3+ T cells, whereas 6 patients did not (ref. 21; J. Sun and P. Sharma, unpublished data). For the 6 patients who did not demonstrate significant changes in CD4+CD25+FoxP3+ T cells after anti–CTLA-4 therapy, we noted that the frequency of CD4+CD25+CD127low and CD4+CD25+LAP+ cells also did not significantly change between pre- and posttherapy samples (data not shown). In patients who had an increase in the frequency of CD4+CD25+FoxP3+ T cells after treatment with anti–CTLA-4 (n = 6), we observed a concomitant increase in the frequency of CD4+CD25+CD127low cells.

However, we observed that the frequency of CD4+CD25+LAP+ cells did not increase in 3 patients’ samples (Fig. 4A, representative patient and Fig. 4B, summary data of 3 patients) but did increase in 3 different patients’ posttherapy samples (Fig. 4C, representative patient and Fig. 4D, summary data of 3 patients). In addition, CD4+CD25+CD127low cells from posttherapy samples were incapable of suppressing proliferation of autologous CD4+CD25− T cells, whereas CD4+CD25−LAP+ T cells functioned as Tregs to significantly suppress the proliferation of CD4+CD25− T cells (P < 0.01; Fig. 4E).

These data indicate that although FoxP3 and CD127low expression may change in patients after treatment with immunotherapy, these markers may not accurately reflect a predominantly functional Treg population. However, increased LAP expression correlates with a predominantly functional Treg population in the setting of in vitro activation as well as after treatment with active immunotherapy such as anti–CTLA-4. Therefore, for the purposes of immune monitoring of patients treated with active immunotherapeutic agents such as anti–CTLA-4, CD4+CD25+LAP+ cells, as opposed to CD4+CD25+CD127low cells, allow for appropriate identification and isolation of functional Tregs.

DISCUSSION

The identification of Tregs and the major role that they play in controlling immune responses has made it possible to envision effective cancer immunotherapy strategies that can both enhance effector T-cell responses as well as overcome immunosuppression induced by Tregs. However, the current markers that are being used to define Tregs in patients may not be adequate in allowing for appropriate distinctions between Tregs and activated effector cells in patients treated with immunotherapy. In addition, the gold-standard marker for identifying Tregs is an intracellular marker, FoxP3, which prevents the use of this marker for isolation of viable cells for functional studies.

Furthermore, FoxP3 has been shown to increase in conventional non-Tregs after T-cell activation, which limits the use of this marker to define the impact of certain immunotherapy agents on Tregs. In a previous publication, it was shown that in vitro-activated human T cells acquired FoxP3 expression and after the cells were rested for a period of time, the cells from some donors had a decrease in FoxP3 expression while cells from other donors retained stable FoxP3 expression as well as suppressive function (9). Therefore, it remains unclear whether increased FoxP3 expression in T cells from patients treated with active immunotherapy agents is representative of a transient occurrence or a legitimate Treg population.

Anti–CTLA-4 immunotherapy has been reported to enhance T-cell responses that lead to antitumor responses. Anti–CTLA-4 (ipilimumab) was approved by the FDA in March 2011 as a standard-of-care treatment for patients with metastatic melanoma. Investigations are ongoing to understand the immunologic mechanisms elicited by anti–CTLA-4 that lead to clinical benefit. We previously documented that anti–CTLA-4 (ipilimumab) therapy led to an increased frequency of inducible T-cell costimulator-positive (ICOS+) T cells that consisted of a population of effector T cells (Teff) in both tumor tissues and peripheral blood (20–22). We also documented that the administration of ipilimumab led to a decreased frequency of FoxP3+ Tregs in tumor tissues, thereby leading to a shift in the Teff (ICOS+)/Treg (FoxP3+) ratio within tumor tissues as a result of treatment (20–22).

However, when we attempted to calculate the Teff/Treg ratio in peripheral blood by using the same T-cell markers, we found that the change between pretherapy and posttherapy values for the frequency of FoxP3+ T cells was inconsistent with the changes observed in tumor tissues. Because FoxP3 expression can increase transiently in activated T cells, it is possible that FoxP3 expression in T cells from peripheral blood of patients treated with anti–CTLA-4 does not accurately reflect a predominantly functional Treg population. Therefore, we examined CD127 expression as a surrogate marker of Tregs in peripheral blood. However, as we report here, upon in vitro activation of PBMCs from healthy donors or after patients are treated with anti–CTLA-4 immunotherapy, CD4+CD25+CD127low cells do not accurately represent a predominantly functional Treg population (Figs. 2 and 4).

LAP binds to the cytokine TGF-β to form a latent complex, which is inactive. The complex of LAP plus TGF-β can be expressed on the membrane of many cell types, including megakaryocytes, platelets, immature dendritic cells, and Tregs (18–20). TGF-β is a pleiotropic cytokine and has been reported to play an important role in the function of Tregs (24). There are multiple mechanisms that can lead to the activation of TGF-β, which requires release from LAP.
**Figure 4.** Frequency of CD4⁺CD25⁺FoxP3⁺, CD4⁺CD25⁺CD127⁰, and CD4⁺CD25⁺LAP⁺ T cells before and after treatment with anti-CTLA-4. Representative patient who had an increase in frequency of CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁺CD127⁰ T cells but not in CD4⁺CD25⁺LAP⁺ T cells after treatment with anti-CTLA-4 (posttherapy) as compared with before treatment (pretreatment) (A). Summary data from 3 patients who had an increase in frequency of CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁺CD127⁰ T cells but not in CD4⁺CD25⁺LAP⁺ T cells after treatment with anti-CTLA-4 (post) as compared with before treatment (pre) (B). Representative patient who had an increase in frequency of CD4⁺CD25⁺FoxP3⁺, CD4⁺CD25⁺CD127⁰, and CD4⁺CD25⁺LAP⁺ T cells after treatment with anti-CTLA-4 (posttherapy) as compared with before treatment (pretreatment) (C). Summary data from 3 patients who had an increase in frequency of CD4⁺CD25⁺FoxP3⁺, CD4⁺CD25⁺CD127⁰, and CD4⁺CD25⁺LAP⁺ T cells after treatment with anti-CTLA-4 (post) as compared with before treatment (pre) (D). Representative thymidine incorporation assay, which measured cell proliferation in the presence of media only, CD4⁺CD25⁺LAP⁺ T cells only with a ratio of R:S of 0.1, CD4⁺CD25⁺CD127⁰ cells only with a R:S ratio of 0.1, responder CD4⁺CD25⁺ cells only with a ratio of R:S of 0.1, a mixture of R:S (CD4⁺CD25⁺CD127⁰) at a ratio of 1:1, and a mixture of R:S (CD4⁺CD25⁺LAP⁺) at a ratio of 1:1 (E). Each experiment was performed in triplicate and suppression assays were repeated with 3 different donor samples. Bars represent mean ± SEM.
In this study, we show that LAP can be used as a marker to accurately identify a population of predominantly functional Tregs. More importantly, because LAP is an extra-cellular marker, it can be used to isolate viable cells for additional studies. We found that CD4+CD25+LAP+ T cells were capable of suppressing immune responses in vitro. In both settings of in vitro activation of PBMCs and in vivo anti–CTLA-4 therapy, LAP identified Tregs from peripheral blood. On the basis of these data, we propose that LAP be included as a marker of Tregs for immune monitoring of patients receiving treatment with immunotherapy agents such as anti–CTLA-4. We are conducting studies to determine whether the Teff/Treg ratio as measured by ICOS and LAP can serve as a biomarker for clinical responses after treatment with anti–CTLA-4.

There are extensive ongoing investigations to define Tregs, which may fall into multiple categories, including natural and adaptive Tregs. The different populations that are encompassed by the term “Treg” are evolving as researchers gain a better understanding of how Tregs develop. FoxP3 expression has been reported in CD4+ T/LAP+ T cells even in CD8 T cells (25). In our studies, CD4+CD25+LAP+ T cells do not encompass 100% of FoxP3+ T cells, nor do CD4+CD25+FoxP3+ cells encompass 100% of LAP+ T cells, which raises the question of whether a subset of CD4+CD25+LAP+ T cells consist of FoxP3+ Tregs or conversely whether a subset of CD4+CD25+LAP+ T cells are FoxP3+ Tregs. Additional studies will need to be conducted to answer these questions. However, on the basis of our current studies, we propose that LAP is an appropriate marker to be used for the identification of functional Tregs in patients treated with active immunotherapy agents.

**METHODS**

**Healthy Donors and Patient Samples**

Peripheral blood samples were obtained from 8 healthy adult donors after they provided appropriate informed consent on MD Anderson Cancer Center Institutional Review Board-approved lab protocol 2005-0027. PBMCs were isolated from whole blood by density gradient centrifugation via the use of Lymphocyte Separation Medium (Mediatech, Inc.) and Leucosep tubes (Greiner Bio-One). Blood samples were obtained from patients with bladder cancer who were treated with anti–CTLA-4 after appropriate informed consent was obtained as per Institutional Review Board-approved protocol 2006-0080, as previously published (20–22).

**In Vitro Activation of T Cells**

We coated 25-cm² cell culture flasks (Corning Incorporated) with 33 μL of αCD3 antibody (BD Biosciences; clone HIT3a) plus 6.6 mL of coating buffer (1 carbonate bicarbonate buffer capsules, Sigma-Aldrich, in 100 mL of PBS) for a final αCD3 concentration of 5 μg/mL. Flasks were left overnight at 4°C. The following day, the plates were washed with PBS. Freshly isolated PBMCs were then placed into the flasks for 48 hours. Each flask contained 30 million PBMCs in RPMI 1640 with 10% human AB serum (Invitrogen) and 2 μg/mL of αCD28 antibody (BD Biosciences).

**Flow Cytometry**

Antibodies used for flow cytometry consisted of: CD4 AmCyan and CD25-PECy7 (BD Biosciences); CD127 Pacific Blue and FOXP3 PerCP-Cy 5.5 (eBioscience); and LAP-APC (R&D Systems). Freshly isolated PBMCs and in vitro-activated PBMCs were stained as per manufacturers’ instructions. Samples were analyzed with the FACS Canto II (Becton Dickinson). Data were analyzed with BD FACSDiva software. Gates were set according to appropriate isotype control.

**Suppression Assays**

We coated 96-well U-bottom plates (Corning Incorporated) with 10 μg/mL αCD3 overnight at 4°C. The plates were then washed with PBS and 5 × 10⁵ CD4+CD25− responder (R) cells; in addition, CD4+CD25+CD127high or CD4+CD25+LAP+ cells were added at indicated ratios. Cells were left in culture for 48 hours in RPMI 1640 plus 10% human AB serum. Then, 1 μCi/well tritiated thymidine (Morawek Biochemicals) was added for the last 18 hours of culture. Thymidine incorporation was measured with the use of TopCount NXT Microplate Scintillation and Luminescence Counter (Packard).

For carboxyfluorescein diacetate succinimidyl ester assays, PBMCs were labeled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester ( Molecular Probes/Invitrogen). CFSE-labeled CD4+CD25−, CD4+CD25+ (CD127high) and CD4+CD25+LAP+ cells were then sorted. A total of 5 × 10⁵ cells were co-cultured for a 1:1 ratio of Treg/target cells per well of a 96-well plate, which was previously coated with 10 μg/mL αCD3. Culture medium was RPMI 1640 with 10% human AB serum. Three days later, carboxyfluorescein diacetate succinimidyl ester dilution was analyzed by flow cytometry.

**Statistical Analysis**

All group results are expressed as mean ± SD, if not stated otherwise. The paired Student t test was used for comparison of group values and discriminatory parameters, where appropriate. P values less than 0.05 were considered significant.

**Disclosure of Potential Conflicts of Interest**

P. Sharma has served on BMS advisory boards and received honoraria for her services. No potential conflicts of interest were disclosed by the other authors.

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