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# CD4<sup>+</sup>CD25<sup>int</sup> T Cells in Inflammatory Diseases Refractory to Treatment with Glucocorticoids<sup>1</sup>

Richard W. J. Lee,\* Thomas J. Creed,<sup>†‡</sup> Lauren P. Schewitz,\* Paul V. Newcomb,<sup>‡</sup> Lindsay B. Nicholson,\*<sup>§</sup> Andrew D. Dick,<sup>2\*§</sup> and Colin M. Dayan<sup>‡</sup>

Up to 30% of patients with autoimmune, allergic, and lymphoproliferative diseases are refractory to glucocorticoid therapy. The present study was undertaken to investigate whether such steroid resistance (SR) is limited to a subpopulation of CD4<sup>+</sup> T cells and, as IL-2 is a putative driver of SR, whether T cell SR is associated with CD25 expression. We show that SR patients have a characteristic subgroup of activated CD4<sup>+</sup> T cells that continue to proliferate despite exposure to high-dose Dexamethasone (Dex), demonstrate that CD4<sup>+</sup>CD25<sup>-</sup> cells are exquisitely sensitive to Dex whereas CD4<sup>+</sup>CD25<sup>int</sup> cells are highly SR, and further find that the combination of an anti-CD25 mAb with Dex enhances suppression of T cell proliferation compared with each agent alone. We therefore conclude that SR is not a general property of all lymphocytes but resides in T cell subpopulations, which are prevalent in SR patients and express intermediary levels of CD25. As a result, we propose a new paradigm for SR disease in which glucocorticoid therapy positively selects SR cells, generating a population of drug-resistant lymphocytes that perpetuate on-going inflammation. *The Journal of Immunology*, 2007, 179: 7941–7948.

Glucocorticoids (GC)<sup>3</sup> remain the first-line treatment for a wide range of autoimmune, allergic, and lymphoproliferative diseases. However, up to 30% of patients with ulcerative colitis (UC), asthma, Systemic Lupus Erythematosus, rheumatoid arthritis, acute lymphoblastic leukemia, and uveitis have a disease that is refractory to GC therapy (1, 2). Such steroid resistance is independent of disease severity and often results in sustained high-dose GC exposure (3). Affected individuals are therefore not only subject to the adverse sequelae of on-going tissue damage and inflammation, but also the systemic adverse effects of GCs, including centripetal obesity, skin atrophy, osteoporosis, diabetes mellitus, hypertension, and mood disturbance.

Inhibition of lymphocyte proliferation by GCs was first demonstrated in 1961(4) and later reports established this was due to suppression of IL-2 production and mRNA expression (5, 6). Impaired in vitro responses to the synthetic GC dexamethasone (Dex) have been observed in PHA stimulated PBMCs from patients with steroid resistant (SR) asthma, SR UC, and SR rheumatoid arthritis, as well as SR renal transplant recipients (7–10). There is now

accumulating evidence that disparities in IL-2 responses to GCs are central to the SR phenotype. IL-2, in combination with IL-4, reduces both glucocorticoid receptor (GR) binding affinity and Dex inhibition of proliferation in PBMCs from normal volunteers (NVs) to levels seen in PBMCs from SR patients (11). It also sustains reduced GR affinity in PBMCs from SR patients, which is reversed on transfer to cytokine-free cell culture medium (11). Furthermore, IL-2 mRNA expression is significantly greater in bronchoalveolar lavage samples from patients with SR asthma than steroid sensitive (SS) controls (12).

IL-2's influence on steroid sensitivity is not limited to modulation of GR binding, as it also inhibits STAT-5-dependent GR translocation into the nucleus in the murine HT-2 cell line (13). In human PBMCs, it has been shown to induce expression of the transcription factor AP-1 (which suppresses GR binding to DNA) as well as the nonbinding GR $\beta$  isoform (which suppresses GR $\alpha$ -mediated GC action) (14, 15). Both AP-1 and GR $\beta$  are reportedly increased in patients with SR asthma, (15, 16) and GR $\beta$  expression is also increased in PBMCs from SR patients with UC (17). Other potential mediators of clinically significant IL-2/GR interactions include FoxO3 (a forkhead transcription factor responsible for IL-2 inhibition of the GC-inducible gene, *giz*), histone deacetylase 2 (via the effect of deacetylated GR on NF- $\kappa$ B-dependent gene expression), and GR gene polymorphisms (which may alter GR-induced regulation of I $\kappa$ B $\alpha$ ). (18–20) Although, disparities in these between SS and SR patients have not yet been demonstrated.

The experimental evidence for the role of IL-2 in SR disease has been further supported by the results of a recent phase II trial of a mAb to the  $\alpha$ -subunit of the IL-2 receptor (CD25), which demonstrated clinical rescue in SR UC (21). We therefore hypothesized that the SR phenotype is not a feature of all PBMCs in SR patients, but rather is limited to CD4<sup>+</sup> T cells that express CD25. A proposal that is also consistent with recent murine evidence that CD4<sup>+</sup>CD25<sup>+</sup> T cells are preferentially spared after systemic GC exposure (22).

In humans, the T regulatory (Treg) cells responsible for inhibiting immune responses and maintaining self-tolerance predominantly reside in the 2% of CD4<sup>+</sup> cells with greatest CD25 expression (CD25<sup>high</sup>) (23). However, it is unlikely that these CD25<sup>high</sup>

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<sup>3</sup> Abbreviations used in this paper: GC, glucocorticoid; SR, steroid resistant; GR, glucocorticoid receptor; SS, steroid sensitive; Dex, dexamethasone; UC, ulcerative colitis; Treg, T regulatory cell; NV, normal volunteer; Bas, basiliximab; 7-AAD, 7-aminoactinomycin; 5-ASA, 5-aminosalicylic acid; int, intermediate.

Table I. Characteristics of study subjects used for case-control comparisons

Participant	Age (years)	Sex <sup>a</sup>	Treatment
SR1	43	M	None
SR2	39	F	None
SR3	58	M	5-ASA
SR4	51	M	5-ASA
SR5	29	M	None
SR6	21	F	5-ASA
NV1	44	M	None
NV2	63	M	None
NV3	58	F	None
NV4	32	M	None
NV5	37	M	None
NV6	24	M	None
NV7	42	F	None
NV8	26	F	None
NV9	28	M	None
NV10	33	F	None
NV11	23	M	None
NV12	41	M	None
NV13	50	F	None
NV14	44	M	None
NV15	27	M	None

<sup>a</sup> M, Male; F, female.

Treg cells mediate the benefits of anti-CD25 mAb therapy observed in SR UC as their regulatory function is unaffected by anti-CD25 mAbs *in vitro* (24). We therefore propose that the influence exerted by CD25 on steroid sensitivity resides in the remaining CD4<sup>+</sup> cells with lower levels of CD25 expression (CD25<sup>int</sup>). We postulate that in SR patients, CD4<sup>+</sup>CD25<sup>int</sup> cells are less responsive to the inhibitory effects of GCs, enabling them to continue proliferating and perpetuating disease.

The current study uses flow cytometric techniques to identify a subpopulation of CD4<sup>+</sup> T cells that are resistant to the anti-proliferative effects of GCs and are prevalent in patients with clinically defined SR UC. We further demonstrate that CD4<sup>+</sup>CD25<sup>int</sup> cells are resistant to GC inhibition in contrast to CD4<sup>+</sup>CD25<sup>-</sup>

cells, which are exquisitely sensitive, and show that anti-CD25 mAbs abrogate sustained CD4<sup>+</sup> T cell proliferation in the presence of GCs.

## Materials and Methods

### Study participants

Twenty-three NVs and six patients with a previous history of SR UC participated. A previous episode of SR UC was defined as either (i) ongoing disease activity despite corticosteroid treatment requiring colectomy, (ii) active colitis (UC Symptom Score  $\geq 6$ ) (25) despite at least 2 wk of prednisone  $\geq 30$  mg per day, or (iii) Truelove and Witts measures of disease severity (26) with poor indicators of outcome (27) despite at least 3 days of *i.v.* steroids. All SR patients had been off systemic GC treatment for at least 3 mo before enrollment. Three had previously participated in a randomized control trial of anti-CD25 mAb therapy in SR UC (a minimum of 1 year before participation in this study), in which they received a single *i.v.* infusion of 40 mg Basiliximab (Bas) (21).

This study was reviewed and approved by the Central and South Bristol Local Research Ethics Committee (Reference no. 04/Q2006/163).

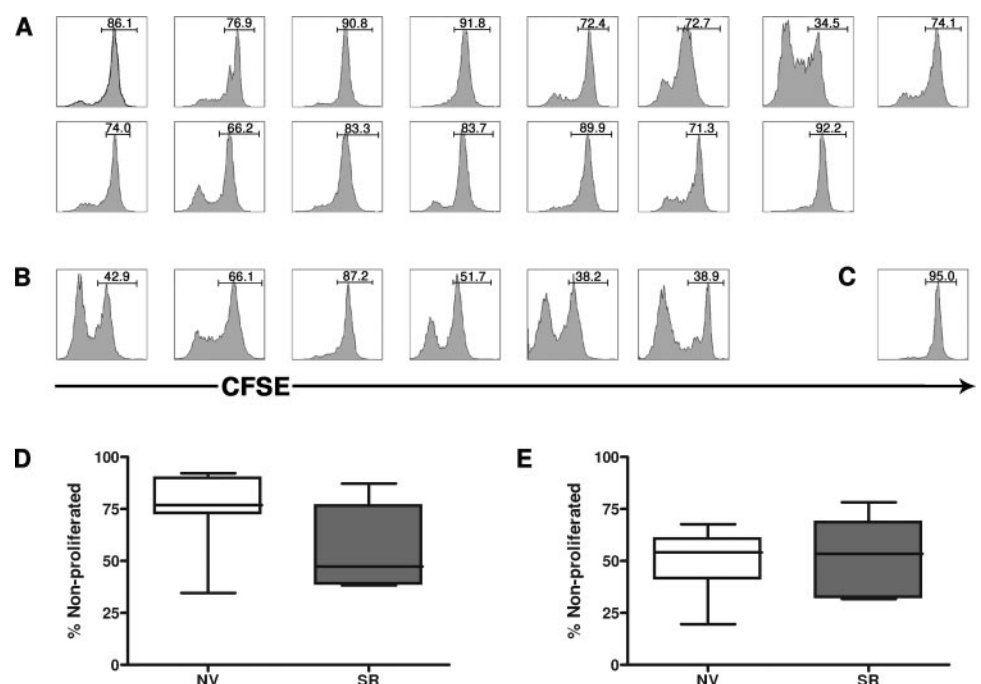
### PBMC preparation and culture

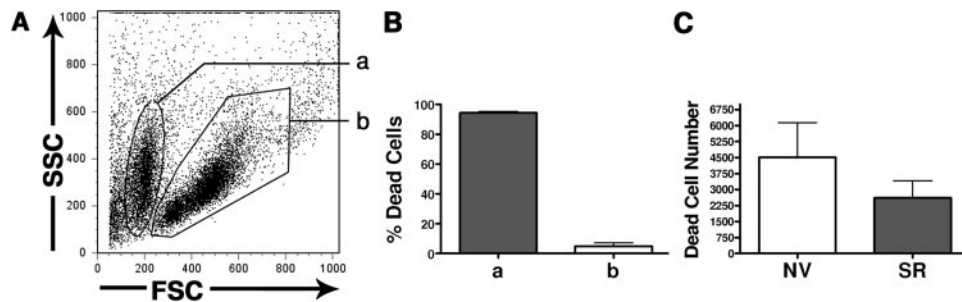
PBMCs were isolated from whole blood on a density gradient (Ficoll-Paque PLUS, GE Healthcare) and stained with CFSE (Vybrant CFDA SE cell tracer kit (V-12883); Molecular Probes) before culture in RPMI 1640 with 10% heat-inactivated FBS (Invitrogen Life Technologies), 100 IU/ml penicillin G, 100 mg/ml streptomycin, and 20 mM HEPES buffer (Invitrogen Life Technologies). Cells were seeded in 96-well U-bottom plates ( $1 \times 10^5$  per well) and incubated at 37°C in humidified air with 5% CO<sub>2</sub> in the presence of  $10^{-10}$ M- $10^{-6}$ M Dex (Sigma-Aldrich), 1  $\mu$ g/ml Bas (a chimeric anti-CD25 mAb; Novartis Pharmaceuticals), or 100 ng/ml Tacrolimus (a calcineurin inhibitor; Astellas Pharma). CD3CD28 coated Dynabeads (DynaL Biotech) were then added (2  $\mu$ l per well) to stimulate T cell proliferation.

### Preculture CD3CD25 phenotyping

A separate sample of fresh PBMCs was stained with PE-conjugated anti-CD25 (Miltenyi Biotec, 1/10 dilution) and PE-alexa fluor 610-conjugated anti-CD3 (Caltag Laboratories, 1/40 dilution). Preculture CD3<sup>+</sup>CD25 expression was then determined by flow cytometry (FACSCalibur; BD Biosciences) and analyzed using CellQuest software.

**FIGURE 1.** CD3<sup>+</sup>CD4<sup>+</sup> cell CFSE proliferation profiles in PBMC cultures exposed to  $10^{-6}$ M Dex. The marker in the top right-hand corner of each graph indicates the proportion of nonproliferating cells. The proportion of nonproliferating cells is the percentage of cells that have the same CFSE fluorescence as 95% of cells in parallel cultures maximally suppressed with 100 ng/ml Tacrolimus. A, NVs,  $n = 15$ . B, SR patients,  $n = 6$ . C, Representative CFSE fluorescence in a control culture maximally suppressed with 100 ng/ml Tacrolimus (PBMCs cultured in the absence of CD3CD28 Dynabeads, *i.e.*, in the presence of medium and serum alone, also did not proliferate). D, Box-plots of grouped data from A and B, comparing the proportion of nonproliferating cells in NV and SR cultures exposed to  $10^{-6}$ M Dex ( $p = 0.03$ ). E, Box-plots of the proportion of nonproliferating cells in NV and SR cultures without Dex, demonstrating equivalent uninhibited division.





**FIGURE 2.** PBMC death after 5 days culture in the presence of  $10^{-6}$ M Dex and CD3CD28 beads. *A* and *B*, 7-AAD positive dead cells (*a*) and 7-AAD negative live cells (*b*) have characteristic forward/side-scatter profiles ( $n = 4$ ). The additional proportion of annexin V positive apoptosing cells in gate *b* is  $<5\%$ . *C*, Comparison of dead cell numbers between NVs ( $n = 15$ ) and SR ( $n = 6$ ) patients, expressed as the number of cells in gate *a* per 10,000 cells in gate *b* ( $p = 0.85$ ) (CD3<sup>+</sup>CD4<sup>+</sup> cell CFSE proliferation profiles for these subjects are shown in Fig. 1).

### CD3<sup>+</sup>CD4<sup>+</sup> cell proliferation studies

PBMCs were washed after 5 days culture and stained with PE-conjugated anti-CD4 (BD Pharmingen, 1/100 dilution) and allophycocyanin-conjugated anti-CD3 (BD Pharmingen, 1/100 dilution). CFSE dilution in CD3<sup>+</sup>CD4<sup>+</sup> cells was then quantified by flow cytometry (28).

### Quantification of apoptosis and cell death

The forward and side-scatter characteristics of apoptosing and dead cells, after 5 days culture in the presence of  $10^{-6}$ M Dex, were determined in four NVs using annexin V-FITC (BD Biosciences) and 7-aminoactinomycin (7-AAD; BD Biosciences). These forward and side-scatter profiles were then used as a proxy measure of the proportion of viable and nonviable cells in subsequent cultures.

### Comparison of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>int</sup> cell responses to Dex

PBMCs were labeled with PE-conjugated anti-CD25 and PE-Alexa Fluor 610-conjugated anti-CD3 and sorted on a FACSVantage to isolate CD3<sup>+</sup>CD25<sup>-</sup> and CD3<sup>+</sup>CD25<sup>int</sup> T cells, achieving  $>80\%$  purity (the 2.5% of cells with greatest CD25 expression were designated CD25<sup>high</sup>, and the remaining CD25<sup>+</sup> cells were defined as CD25<sup>int</sup>). Each population was then separately stained with CFSE and cultured in the same conditions described above for PBMCs. After 5 days, cultured cells were washed and labeled with PE-conjugated anti-CD4. CFSE dilution in CD4<sup>+</sup> cells was then determined by flow cytometry.

### Determination of the effect of inducing “intermediary” expression of CD25 on CD4<sup>+</sup> cell responses to Dex

CD4<sup>+</sup>CD25<sup>-</sup> cells were isolated using magnetic beads (achieving  $>90\%$  purity; CD4<sup>+</sup> T cells were first positively selected and then depleted of CD25<sup>+</sup> cells (Miltenyi Biotec, CD4<sup>+</sup> T cell isolation kit II and CD25 MicroBeads II kit; both used in accordance with the manufacturer’s instructions) and then either stained with CFSE and cultured in the same conditions described above for PBMCs, or seeded into a 96-well U-bottom plate ( $1 \times 10^5$  cells per well) and activated with 0.25  $\mu$ l per well CD3CD28 coated Dynabeads to induce “intermediate” level CD25 expression in  $>75\%$  of cells. After 24 h, the activated cells were washed free of Dynabeads, stained with CFSE, and cultured for 5 days in the same conditions. CFSE dilution was then determined by flow cytometry and the proliferation of the unactivated vs activated cells was compared.

### Preculture CD4<sup>+</sup>CD25<sup>int</sup> cell activation status and Treg marker expression studies

PBMCs were labeled with selected combinations of the following Abs to determine the preculture activation status and Treg marker expression of CD4<sup>+</sup>CD25<sup>int</sup> cells using four-color flow cytometry (FACSCalibur; BD Biosciences): PE-conjugated anti-CD25 (Miltenyi Biotec; 1/10 dilution), PE-Alexa Fluor 610-conjugated anti-CD3 (Caltag Laboratories; 1/40 dilution), FITC-conjugated anti-CD69 (BD Pharmingen; 1/50 dilution), allophycocyanin-conjugated anti-HLA-DR (BD Pharmingen; 1/100 dilution), allophycocyanin-conjugated anti-CD4 (BD Pharmingen; 1/100 dilution), biotinylated anti-CD44 (BD Pharmingen; 1/100 dilution), FITC-conjugated anti-CD62L (BD Pharmingen; 1/200 dilution), FITC-conjugated anti-CD45RO (Caltag Laboratories; 1/100 dilution), biotinylated anti-CD127 (BD Pharmingen; 1/10 dilution), Alexa Fluor 750-R-PE streptavidin

(Caltag Laboratories; 1/200 dilution), and FITC-conjugated anti-FoxP3 staining set (eBioscience, used in accordance with manufacturers instructions).

NVs whose CD4<sup>+</sup> cells were SS ( $>70\%$  nonproliferating cells) on exposure to  $10^{-6}$ M Dex (see Results below) and SR patients with a clearly defined subpopulation of dividing (CFSE<sup>low</sup>) CD4<sup>+</sup> cells despite exposure to  $10^{-6}$ M Dex were selected for case-control comparisons.

### Flow cytometric and statistical analyses

All flow-cytometric analyses (including proliferation studies) were conducted using FlowJo software (Tree Star). Cells exposed to 100 ng/ml Tacrolimus did not proliferate and a marker set on this population was then applied to all other samples to determine their proportion of nonproliferating cells. All statistical comparisons were calculated using Mann-Whitney tests in GraphPad Prism v. 4.00 for Windows (GraphPad Software) unless otherwise stated.

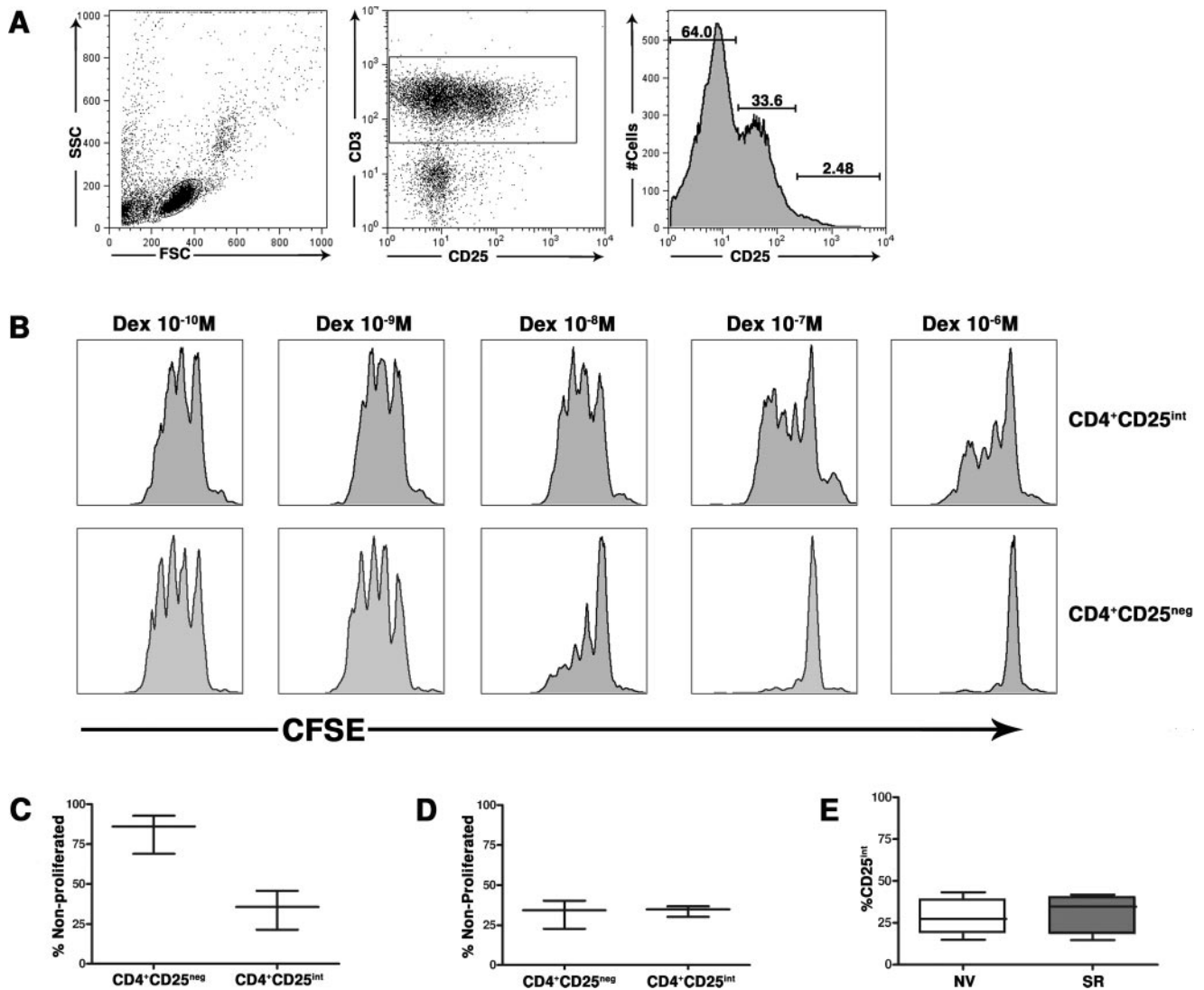
## Results

### Characteristics of study subjects

The mean age of SR patients and NVs used for case-control comparisons was 40 years (range, 21–58 years) and 38 years (range, 23–63 years), respectively. Sex distribution was equal between groups (2/3 male, 1/3 female), and all study participants were Caucasian. All the NVs and three of the SR patients, who had colectomies, were not taking any form of immunosuppressive therapy. The remaining SR patients were all in disease remission, maintained with 5-aminosalicylic acid (5-ASA) alone (Table I).

### CD3<sup>+</sup>CD4<sup>+</sup> cell proliferation in PBMC cultures exposed to high dose Dex

Fig. 1 illustrates the different CFSE profiles of Dex-treated CD3<sup>+</sup>CD4<sup>+</sup> cells from NVs (Fig. 1*A*) and SR patients (Fig. 1*B*). All cultures had a proportion of weakly fluorescent (CFSE<sup>low</sup>) cells that had undergone multiple divisions, but the size of this proliferating subpopulation was greater in SR patients. Cell division was inversely quantified by calculating the “percentage of nonproliferating cells,” which is the proportion of cells with the same CFSE fluorescence as 95% of cells in parallel cultures maximally suppressed with 100 ng/ml Tacrolimus (Fig. 1*C*). This revealed two categories of CFSE profile: those in which  $>70\%$  of cells were undivided and those in which  $<70\%$  of cells were undivided. Only one (16.7%) SR patient had greater than 70% inhibition of proliferation vs 13 (86.7%) NVs (median 47.3% undivided cells in SR patients and 76.9% in NVs,  $p = 0.03$ , Fig. 1*D*). Furthermore, all cultures with  $<70\%$  undivided cells had a characteristic, prominent CFSE<sup>low</sup> peak. This was neither an artifact of variable preculture CFSE staining (as undivided control cells exposed to Tacrolimus had uniformly high fluorescence, Fig. 1*C*) nor variable T cell activation (as CFSE dilution in uninhibited cells was equivalent in all study subjects, Fig. 1*E*). In SR patients, the



**FIGURE 3.** Characteristics of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>int</sup> cells. *A*, *Left panel*: forward/side-scatter profile of unactivated PBMCs with gate set around lymphocytes; *middle panel*: CD3CD25 profile of gated lymphocytes; *right panel*: histogram of CD25 expression in CD3<sup>+</sup> cells, left-hand peak = CD25<sup>-</sup> cells, central peak = CD25<sup>int</sup> cells, right-hand tail = CD25<sup>high</sup> cells. *B*, Representative CFSE proliferation profiles of CD4<sup>+</sup>CD25<sup>int</sup> (*top row*) and CD4<sup>+</sup>CD25<sup>-</sup> (*bottom row*) cells isolated by FACS Vantage sort from NVs ( $n = 3$ ) PBMCs and cultured in the presence of increasing concentrations of Dex (columns). *C*, Box-plots comparing the proportion of nonproliferating cells (see Fig. 1 legend for definition) in CD25<sup>-</sup> and CD25<sup>int</sup> cultures exposed to 10<sup>-6</sup>M Dex (NV,  $n = 3$ ). *D*, Box-plots of the proportion of nonproliferating cells in parallel CD25<sup>-</sup> and CD25<sup>int</sup> cultures without Dex, demonstrating equivalent uninhibited division (NV,  $n = 3$ ). *E*, Box-plot of the proportion of CD4<sup>+</sup>CD25<sup>int</sup> cells in unactivated PBMCs from NVs ( $n = 10$ ) compared with SR patients ( $n = 6$ ),  $p = 0.71$  (CD3<sup>+</sup>CD4<sup>+</sup> cell CFSE proliferation profiles for these subjects are shown in Fig. 1).

presence of a prominent CFSE<sup>low</sup> peak was also unrelated to 5-ASA use ( $p = 1.0$ , Fisher's exact test). The number of dead and apoptosing cells (identified by their characteristic forward and side-scatter profile on flow cytometry, Fig. 2, *A* and *B*) after 5 days culture in the presence of 10<sup>-6</sup>M Dex was less in SR patients compared with NVs. However, this difference did not reach statistical significance (Fig. 2*C*).

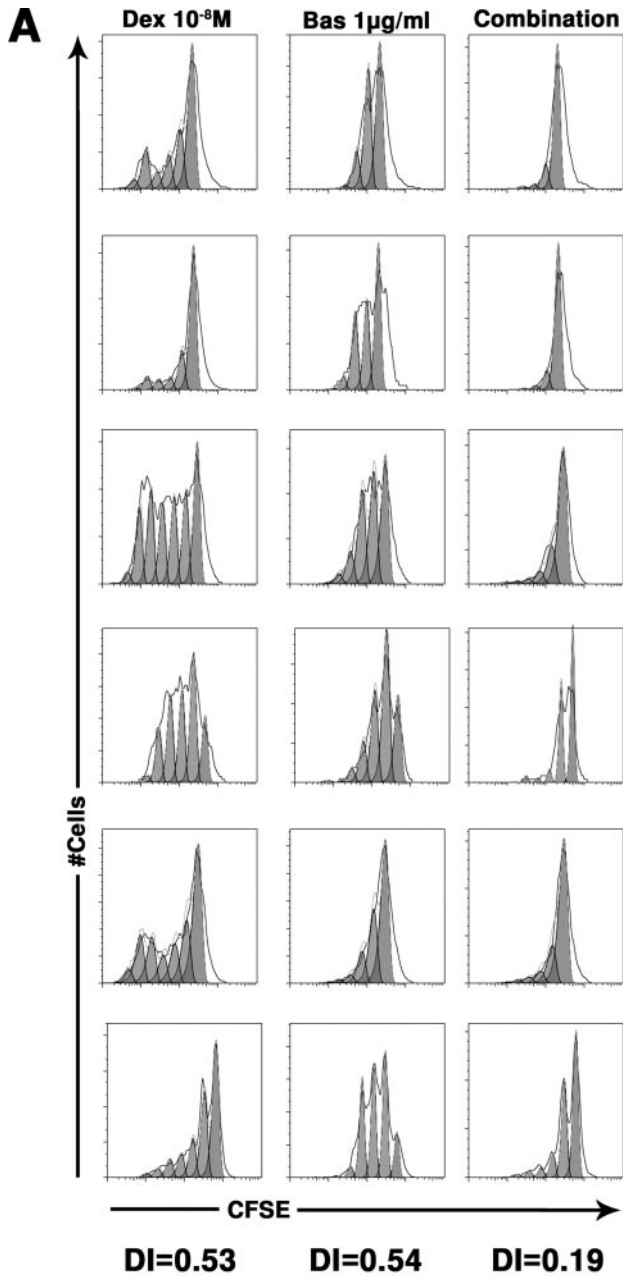
#### Differential proliferation of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>int</sup> cells exposed to high dose Dex

A typical preculture CD25 expression profile is shown in Fig. 3*A* (see figure legend for definition of CD25<sup>-</sup>, CD25<sup>int</sup>, and CD25<sup>high</sup> cells). Fig. 3*B* shows the CFSE proliferation profiles of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>int</sup> cells that had been isolated by flow cytometry and exposed to increasing concentrations of Dex. Both CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>int</sup> cells proliferated equally well in the presence of subtherapeutic (10<sup>-10</sup>M) Dex, but

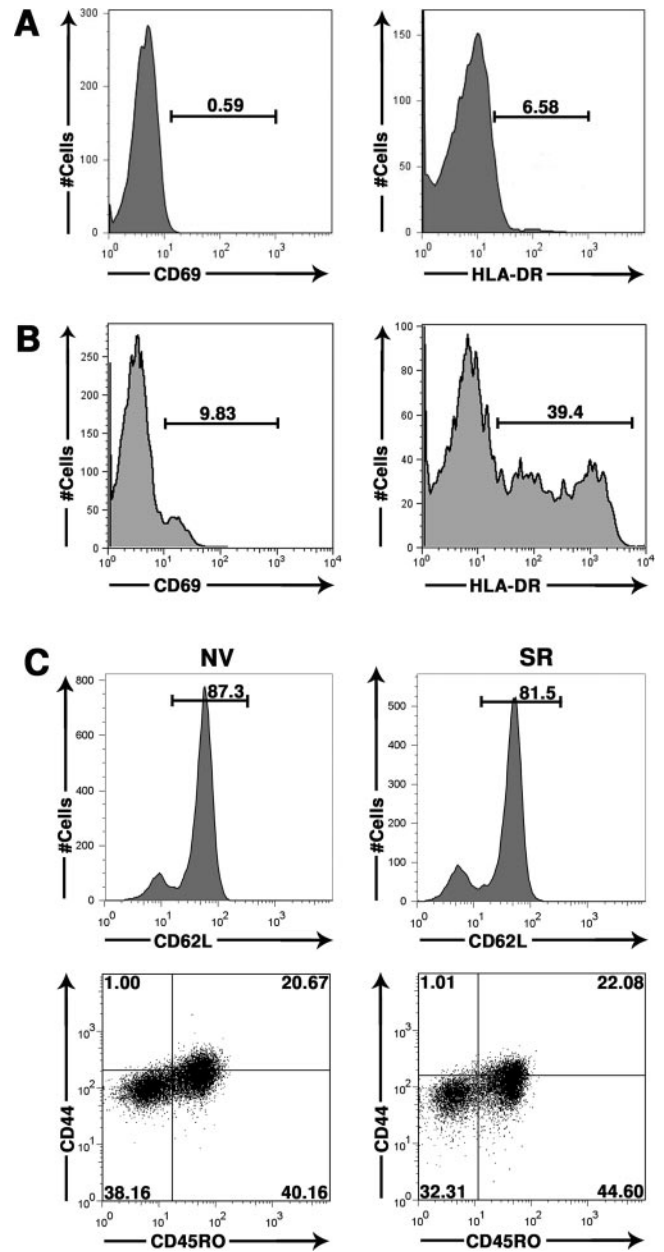
CD4<sup>+</sup>CD25<sup>-</sup> cells were very sensitive to increasing GC concentrations with complete abrogation of CFSE dilution at 10<sup>-7</sup>M Dex. In contrast, CD4<sup>+</sup>CD25<sup>int</sup> cells continued to proliferate even in the presence of 10<sup>-6</sup>M Dex (Fig. 3*B*). The same result was replicated on samples from three different NVs (median 86.0% undivided CD4<sup>+</sup>CD25<sup>-</sup> cells vs 35.6% undivided CD4<sup>+</sup>CD25<sup>int</sup> cells, Fig. 2*C*). CFSE dilution in uninhibited CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>int</sup> cells was equal (Fig. 3*D*), confirming that the difference in these two subpopulations is not secondary to a disparity in the degree of T cell activation. Although the median proportion of CD4<sup>+</sup>CD25<sup>int</sup> cells in fresh PBMCs from SR patients was greater than in PBMCs from NVs (34.6% vs 27.4%), this was not statistically significant (Fig. 3*E*).

#### Effect of low dose Dex combined with anti-CD25 mAbs on CD4<sup>+</sup> cells

To determine whether the level of CD25 expression in CD25<sup>int</sup> cells was functional, we explored the effects of CD25 inhibition in



**FIGURE 4.** CD3<sup>+</sup>CD4<sup>+</sup> cell CFSE proliferation profiles in PBMC cultures exposed to 10<sup>-8</sup>M Dex and 1 µg/ml Bas. *A*, Each row represents the proliferative response of activated CD3<sup>+</sup>CD4<sup>+</sup> cells from a single NV in the presence of Dex alone (*left panels*), Bas alone (*middle panels*), and Dex and Bas combined (*right panels*). Computed division peaks are superimposed. The division index is the average number of divisions each cell has undergone (mean value for all cultures in each column shown, *n* = 6). *B*, Bar chart of division indexes for each column in *A* (*n* = 6), *p* = 0.02 (Dex vs Dex and Bas combined).



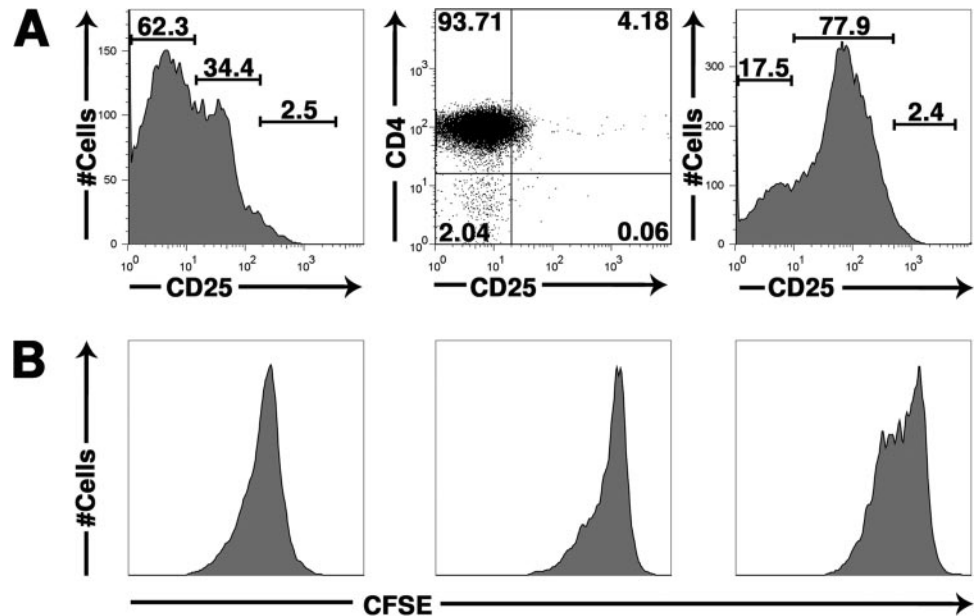
**FIGURE 5.** Activation marker expression. *A*, Representative NV CD69 and HLA-DR profile (*n* = 9) in preculture CD4<sup>+</sup>CD25<sup>int</sup> cells. *B*, Expression of CD69 and HLA-DR in control PBMC 48 h after in vitro CD3CD28 activation. *C*, Representative CD62L and CD44<sup>high</sup> expression in preculture CD4<sup>+</sup>CD25<sup>int</sup> cells from NVs (*n* = 2) and SR patients (*n* = 2). The marker for discriminating CD44<sup>high</sup> and CD44<sup>low</sup> was set at a level where all but 1% of RO-negative cells were assigned as CD44<sup>low</sup> (30–32). CD4<sup>+</sup> cells from the NVs used for this comparison had a SS CFSE profile (and from the SR patients had a SR CFSE profile) on exposure to 10<sup>-6</sup>M Dex (see Fig. 1).

combination with 10<sup>-8</sup>M Dex. CFSE dilution in CD3<sup>+</sup>CD4<sup>+</sup> cells from NVs was only partially suppressed on exposure to either 10<sup>-8</sup>M Dex or 1 µg/ml Bas (equivalent to therapeutic serum levels) alone (Fig. 4*A*). However, when Dex and Bas were combined the remaining peak of rapidly proliferating CFSE<sup>low</sup> cells was lost (Fig. 4*A*), significantly reducing the average number of cell divisions (*p* = 0.02, Fig. 4*B*).

*Phenotypic and functional analysis of CD4<sup>+</sup>CD25<sup>int</sup> cells*

Despite their CD25 expression, fresh, unstimulated CD4<sup>+</sup>CD25<sup>int</sup> cells did not express either the early activation marker CD69 or the

**FIGURE 6.** The effect of inducing “intermediate” expression of CD25 on CD4<sup>+</sup> cell responses to Dex. *A, Left panel*, histogram of CD25 expression in preculture CD4<sup>+</sup> cells; *middle panel*, dot-plot of CD4CD25 expression after isolation of CD4<sup>+</sup>CD25<sup>int</sup> cells using magnetic beads (achieving >90% purity); *right panel*, histogram of CD25 expression in CD4<sup>+</sup>CD25<sup>int</sup> cells 24 h after activation with CD3CD28 coated Dynabeads (>75% express an “intermediate” level of CD25). *B*, Representative NV CFSE proliferation profiles (*n* = 2). *Left panel*, CD4<sup>+</sup>CD25<sup>int</sup> cells cultured in the presence of 10<sup>-6</sup>M Dex; *middle panel*, CD4<sup>+</sup>CD25<sup>int</sup> cells activated to express an “intermediate” level of CD25 and cultured in the presence of 10<sup>-6</sup>M Dex; *right panel*, CD4<sup>+</sup>CD25<sup>int</sup> cells activated to express an “intermediate” level CD25 and cultured without Dex.



late activation marker HLA-DR. Representative FACS profiles are shown in Fig. 5A, together with the CD69 and HLA-DR profiles of control PBMCs 48 h after activation with CD3CD28 coated Dynabeads (Fig. 5B). The proportion of CD62L<sup>-</sup> and CD45RO<sup>+</sup>CD44<sup>high</sup> cells was also equivalent in CD4<sup>+</sup>CD25<sup>int</sup> cells from NV and SR patients (Fig. 5C). To further determine whether the SR phenotype of CD4<sup>+</sup>CD25<sup>int</sup> was secondary to activation, we induced an “intermediate” level of CD25 expression by activating CD4<sup>+</sup>CD25<sup>int</sup> cells with CD3CD28 coated Dynabeads before culture. These cells were equally susceptible to Dex inhibition of proliferation as unactivated CD4<sup>+</sup>CD25<sup>int</sup> cells (Fig. 6). Furthermore, there was no significant difference in FoxP3 or CD127 expression in CD4<sup>+</sup> cells from NVs and SR patients, either overall or in the CD25<sup>int</sup> subset (Fig. 7).

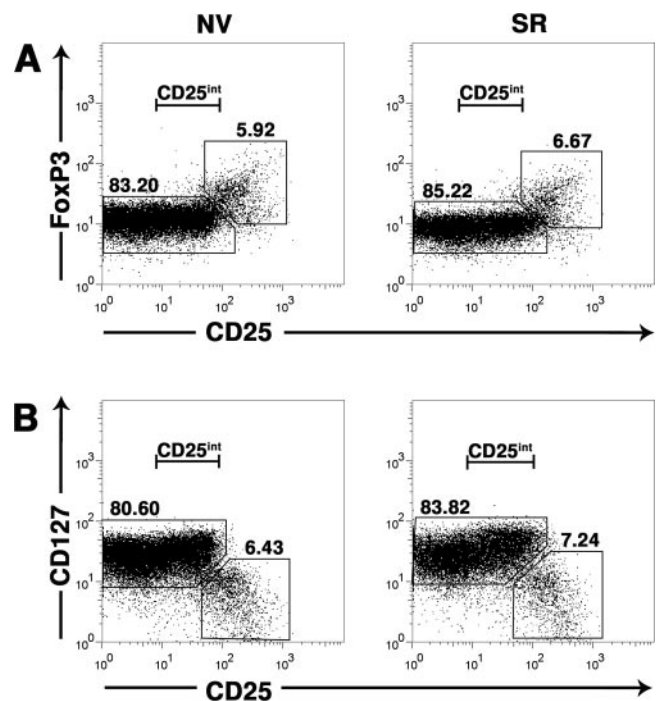
## Discussion

This study establishes a paradigm for steroid resistance based on a subpopulation of CD4<sup>+</sup> T cells that continue to proliferate despite the presence of high dose GCs. The proportion of these SR CD4<sup>+</sup> T cells was significantly greater in patients with previous SR disease (Fig. 1C), and their characteristically low CFSE fluorescence implies that SR CD4<sup>+</sup> cells not only continue to proliferate in the presence of GCs, but proliferate vigorously. If these in vitro findings truly reflect the behavior of SR cells in vivo, a disease model can be envisaged in which SR cells are positively selected, generating a population of drug-resistant lymphocytes which perpetuate on-going inflammation.

The ability of Dex-exposed CD4<sup>+</sup> cells from SR patients to proliferate when in disease remission implies that their SR-phenotype is stable over time and corroborates previous clinical evidence that SR is independent of disease severity (5). Indeed, SR may be unrelated to the presence of disease at all. Seventeen percent of our NVs had a SR phenotype, and this is consistent with previous reports of varying in vitro steroid sensitivity in healthy individuals, adding weight to the theory that SR is an inherent and stable characteristic of an individual which is only manifest in the context of GC therapy (5, 29).

The interpretation of our data is potentially confounded by 5-ASA treatment in the SR group. However, only three (50%) patients were taking 5-ASA, and this did not correlate with their proportion of CFSE<sup>low</sup> CD4<sup>+</sup> cells. It is also counterintuitive to

suggest that prior in vivo T cell exposure to 5-ASA would promote greater proliferation. In addition, three (50%) SR patients had received Bas as part of a previous clinical trial, but as Bas has been shown to increase steroid sensitivity in vitro (21) and the patients' last Bas dose was at least 12 mo before participation in this study, it is unlikely that this has influenced our findings. Furthermore, there was no age, sex, or racial discrepancy between the SR or



**FIGURE 7.** Representative NV (*n* = 2) and SR patient (*n* = 2) CD4<sup>+</sup> cell Treg cell marker expression. *A*, Expression of CD25 and FoxP3 in preculture CD4<sup>+</sup> cells; 5.8% (mean) of NV CD4<sup>+</sup>CD25<sup>int</sup> cells are FoxP3<sup>+</sup> vs 8.0% of SR CD4<sup>+</sup>CD25<sup>int</sup> cells. *B*, Expression of CD25 and CD127 in preculture CD4<sup>+</sup> cells; 7.6% (mean) of NV CD4<sup>+</sup>CD25<sup>int</sup> cells are CD127<sup>low</sup> vs 10.6% of SR CD4<sup>+</sup>CD25<sup>int</sup> cells. CD4<sup>+</sup> cells from the NVs used for this comparison had a SS CFSE profile (and from the SR patients had a SR CFSE profile) on exposure to 10<sup>-6</sup>M Dex (see Fig. 1).

control study groups. Hence, it appears justifiable to attribute their different CFSE profiles to differences in their Dex response.

It is also important to emphasize that the apparently large populations of CFSE<sup>low</sup> cells in cultures from SR patients may have arisen from a tiny subgroup of preculture CD4<sup>+</sup> cells. By basing our statistical comparisons on the proportion of nonproliferating cells, we are highlighting differences in the relative size of proliferating and nonproliferating populations. Consequently, the number of dividing cells may appear greater as a simple consequence of increased apoptosis and cell death in the undivided group. The trend toward lower numbers of dead cells in PBMC cultures from our SR patients (Fig. 2C) suggests that this is not the case. However, more accurate quantification of the dynamics of cell death and apoptosis over the full culture period is needed to properly validate this assertion, as well as to establish whether SR CD4<sup>+</sup> cells from SR patients are also resistant to GC-induced apoptosis.

The continued proliferation of CD4<sup>+</sup>CD25<sup>int</sup> cells in the presence of 10<sup>-6</sup>M Dex, combined with the exquisite sensitivity of CD4<sup>+</sup>CD25<sup>-</sup> cells to 10<sup>-7</sup>M Dex (Fig. 3B), suggests that the SR CD4<sup>+</sup> cells seen in our PBMC cultures arose from the CD4<sup>+</sup>CD25<sup>int</sup> population. This is further corroborated by the complete suppression of proliferation in cells escaping low-dose (10<sup>-8</sup>M) Dex inhibition in the presence of CD25 blockade. However, the similar number of CD25<sup>int</sup> cells in NVs and SR subjects implies that CD25<sup>int</sup> expression alone does not account for the SR phenotype (Fig. 3E), and we therefore speculate that the CD4<sup>+</sup> SR cells we have demonstrated represent only a fraction of the CD25<sup>int</sup> group.

One potential explanation for continued cell division in CD4<sup>+</sup>CD25<sup>int</sup> cells despite exposure to GCs is that they are simply activated and already committed to proliferate. However, this is not substantiated by their CD69 and HLA-DR expression (Fig. 5A) or the fact that CD25<sup>int</sup> cells account for 29% (mean) of circulating CD3<sup>+</sup> cells. Furthermore, the proportion of CD62L<sup>-</sup> and CD45RO<sup>+</sup>CD44<sup>high</sup> cells was equivalent in CD4<sup>+</sup>CD25<sup>int</sup> cells from NV and SR patients (Fig. 5C). The failure to induce SR in T cells activated to express an intermediary level of CD25 before culture (Fig. 6) also mitigates against activation, per se, being the cause of the CD25<sup>int</sup> cell SR we observed.

The association we have made between T cell CD25 expression and an SR phenotype is consistent with previous reports of IL-2-enhanced GR binding affinity and increased IL-2 mRNA expression in SR patients (11, 12). This suggests that SR is secondary to an up-regulation of IL-2, which in turn may reflect altered earlier signaling. For example, induction of the IKBA gene by GCs has been shown to lead to an increased rate of IκBα protein synthesis, hence blocking translocation of NF-κB to the nucleus and consequent IL-2 transcription (33). Such upstream events are therefore candidates for altered GC action in SR T cells, which merit future investigation, as does the potential for their restriction to the CD4<sup>+</sup>CD25<sup>int</sup> cell subpopulation. It is also possible that the SR phenotype is influenced by Treg cell function, but we did not find a significant difference in CD4<sup>+</sup> cell FoxP3 or CD127 expression in our NVs and SR patients (Fig. 7). However, the association between SR disease and a failure of induction of Treg cells has recently been identified in a cohort of SR asthma patients (34).

In summary, we have identified a subpopulation of SR CD4<sup>+</sup> cells which are more prevalent in patients with a previous history of SR disease and have shown that CD4<sup>+</sup>CD25<sup>int</sup> cells replicate this SR phenotype. We therefore propose a model of SR disease based on the selection of such SR CD4<sup>+</sup> T cells following GC treatment. In addition, we have provided further in vitro evidence to support the rationale for using anti-CD25 therapies in conjunction with GCs to treat SR disease.

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## Disclosures

Richard W. J. Lee, Thomas J. Creed, Andrew D. Dick, and Colin M. Dayan share revenue from a patent for the use of anti-CD25 Abs in steroid-resistant inflammatory diseases (US patent application number 10/474,071) on which Colin M. Dayan is an inventor. Colin M. Dayan is also a scientific advisor to Cerimon pharmaceuticals, who are conducting clinical studies on the use of Basiliximab (Novartis) in steroid-resistant inflammatory bowel disease and has previously received grant support from Novartis plc (2001–2003). Lauren P. Schewitz, Paul V. Newcomb, and Lindsay B. Nicholson have no financial conflict of interest to declare.

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