Steroid Refractory $CD4^+$ T Cells in Patients with Sight-Threatening Uveitis

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Purpose. A discrete subpopulation of steroid refractory (SR) $CD4^+$ T cells has recently been identified in patients with SR ulcerative colitis (UC). The purpose of this study was to test whether this subpopulation is also present in patients with clinically defined SR uveitis. As interleukin (IL)-2 experimentally mediates the SR phenotype, the combined effects of dexamethasone (Dex) and a range of IL-2 targeting immunosuppressive agents were also investigated.

Methods. Peripheral blood mononuclear cells (PBMCs) from 27 patients with uveitis and 4 normal volunteers were cultured for 5 days with CD3-CD28 beads. In vitro steroid refractivity or responsiveness was determined by the presence or absence of a subpopulation of SR $CD4^+$ cells (as previously reported for UC) that continued to proliferate or not in the presence of Dex. The patients were concurrently classified by a masked investigator as having clinically SR (threshold for disease reactivation, $\geq$10 mg prednisone daily) or steroid sensitive (SS) disease.

Results. There was 78% (21/27) agreement between the in vitro and clinical classifications of SR and SS disease (κ coefficient = 0.56, $P = 0.002$). This finding corresponds to a positive predictive value of 90% and a negative predictive value of 71%. In normal volunteers, basiliximab, daclizumab, and AG490 achieved an equivalent augmentation of $CD4^+$ cell suppression in combination with Dex.

Conclusions. As in UC, patients with SR uveitis have a subpopulation of SR $CD4^+$ cells that are a potential target for intervention with anti–IL-2 therapies, including inhibitors of JAK/STAT signaling. The identification of SR T cells also has potential clinical application as a biomarker for SR disease. (*Invest Ophthalmol Vis Sci.* 2009;50:4273–4278) DOI:10.1167/iovs.08-3152

Intraocular inflammation (uveitis) is estimated to affect up to 115 people per 100,000 in Western populations,[1] just under a quarter of whom will require systemic immunosuppression for sight-threatening disease.[2] Corticosteroids remain the first-line choice of systemic therapy,[3] but their utility is limited by their side effects, which include centripetal obesity, skin atrophy, osteoporosis, diabetes mellitus, hypertension, and mood disturbance.[4] Treatment success is also variable, and it is estimated that up to a third of patients with uveitis are unable to achieve disease control at tolerable corticosteroid doses.[5,6]

Unlike ulcerative colitis (UC) or asthma,[7,8] there are no agreed clinical definitions of steroid refractory (SR) disease for sight-threatening uveitis. The detrimental impact of suboptimal corticosteroid responses on visual function and quality of life of patients with uveitis is nonetheless a significant problem, as is sustained high-dose corticosteroid use.[3] This is reflected in guidance from the Standardization of Uveitis Nomenclature (SUN) working group, which advocates the adoption of steroid-sparing thresholds as the principle measure of second-line immunosuppressive drug efficacy.[9] Although qualitative, achieving a reduction in corticosteroid dose is therefore a key determinant of treatment success, and there is a clear need to understand the mechanisms that continue to drive inflammation in patients in whom corticosteroid taper fails.

A new paradigm for SR disease has recently been proposed, based on preliminary data from patients with UC, in which glucocorticoid (GC) therapy positively selects SR $CD4^+$ T cells that perpetuate ongoing inflammation.[10] The purpose of this study was to test the hypothesis that patients with SR uveitis also have a subpopulation of SR $CD4^+$ T cells. Furthermore, given the evidence corroborating interleukin (IL)-2's mediation of the SR phenotype,[10–15] the effects of a range of immunosuppressive agents that target different points in the generation and action of IL-2 on $CD4^+$ T cells were investigated.

Methods

Study Participants

Twenty-seven patients under the care of the Regional Ocular Inflammation Service at Bristol Eye Hospital for sight-threatening uveitis and four normal volunteers, were enrolled. The study complied with the tenets of the Declaration of Helsinki and was reviewed and approved by the Central and South Bristol Local Research Ethics Committee (Reference no. 04/Q2006/163). Informed consent was obtained from all participants.

Clinical Classification of Steroid Responsiveness

As there is no accepted definition of SR disease in uveitis, the consensus from the SUN working group of a threshold of 10 mg prednisone daily was thus adopted as the standard for distinguishing between patients with SR and those with steroid-sensitive (SS) disease. To ensure that this classification was based on a pure corticosteroid response, independent of the effects of other immunosuppressants, patients' clinical records were retrospectively reviewed to establish the dose of prednisone at which the disease reactivated during their first cycle of corticosteroid monotherapy. If this was $\geq$10 mg daily, the patient was classified as having SR disease, and if it was $<$10 mg daily, the patient was classified as having SS disease.

Our standard corticosteroid treatment regimen is 60 mg prednisone daily for 5 days, 40 mg daily for 1 week, 30 mg daily for 1 week, 20 mg daily for 2 weeks, 15 mg daily for 2 weeks, and 10 mg daily for 1 month. Subsequent taper is in steps of 1 to 2.5 mg daily.

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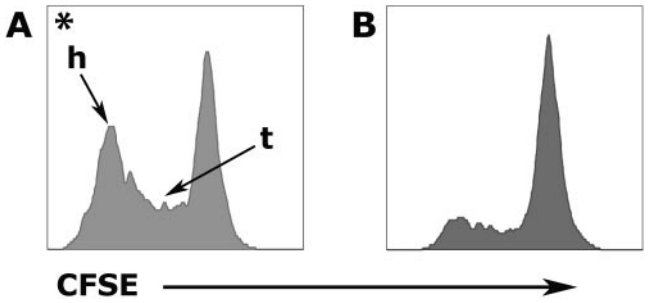

FIGURE 1. In vitro classification of $CD4^+$ cell steroid responsiveness in PBMC cultures exposed to 10^{-6} M dexamethasone. (**A**) An SR carboxyfluorescein succinimidyl ester (CFSE) profile. The SR phenotype (*) was defined by the presence of a discrete subpopulation of $CFSE^{low}$ cells, which had to be separated from neighboring undivided $CFSE^{hi}$ cells by a central trough (t), and have a maximum height (h) at least half that of the $CFSE^{hi}$ population. (**B**) CFSE profiles which did not fulfill this definition were designated SS.

PBMC Preparation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood on a density gradient (Ficoll-Paque Plus; GE Health Care, Piscataway, NJ) and stained with carboxyfluorescein succinimidyl ester (CFSE; Vybrant CFDA SE, Cell Tracer Kit, V-12883; Molecular Probes, Eugene, OR) before culture in RPMI 1640 with 5% heat-inactivated fetal bovine serum (PAA), 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 20 mM HEPES buffer (Invitrogen Life Technologies, Carlsbad, CA). Cells were seeded in 96-well U-bottomed plates (1×10^5 per well) and incubated at 37°C in humidified air with 5% CO_2 in the presence of 10^{-6} M dexamethasone (Dex; Sigma-Aldrich, Poole, UK). CD3-CD28–coated beads (Dynabeads; Dynal Biotech, Oslo, Norway) were then added (0.25 μL/well) to stimulate T-cell proliferation. These culture conditions were optimized for patients with uveitis by titrating the concentration of CD3-CD28 beads used to distinguish between individuals with SR and SS disease, before study enrollment.

$CD4^+$ Cell Proliferation

PBMCs were washed after 5 days in culture and were stained with phycoerythrin-conjugated anti-CD4 (1:400 dilution; Invitrogen Life Technologies,) and allophycocyanin-conjugated anti-CD3 (1:400 dilution; Invitrogen Life Technologies). CFSE dilution in $CD3^+CD4^+$ cells was then quantified by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA) and analyzed (CellQuest software; BD Biosciences).[16] Dead cells were excluded by using 7-aminoactinomycin (7-AAD; BD Biosciences).

In Vitro Classification of Steroid Sensitivity

The CFSE profiles of proliferating $CD4^+$ cells in cultures from patients with uveitis were classified as SR or SS based on the presence or absence of a discrete $CFSE^{low}$ subpopulation, as previously described[10] (Fig. 1).

In Vitro Interleukin-2 Inhibition

PBMCs from four normal volunteers were cultured for 5 days in the presence of 10^{-10} to 10^{-6} M Dex and/or any of the following anti–IL-2 agents: 0.1 ng/mL to 1 μg/mL tacrolimus (a calcineurin inhibitor; Astellas Pharma, Ltd., Staines, UK), 1 ng/mL to 100 μg/mL basiliximab (a chimeric anti-CD25 monoclonal antibody [mAb]; Novartis Pharmaceuticals, Basel, Switzerland), 1 ng/mL to 100 μg/mL daclizumab (a humanized anti-CD25mAb; Roche Pharmaceuticals, Welwyn Garden City, UK), and 0.1 to 30 μg/mL AG490 (a JAK [janus kinase]/STAT [signal transducer and activator of transcription] inhibitor; Calbiochem, San Diego, CA).[17,18] CFSE dilution in $CD3^+CD4^+$ cells was then quantified by flow cytometry (as just described).

Masked Comparison of Clinical and In Vitro Classifications of Steroid Sensitivity and Statistical Analyses

The investigators making the clinical and in vitro classifications of steroid responsiveness were masked to each other's findings. Their degree of agreement was then assessed by calculating the κ coefficient[19] (SPSS ver.14.0 for Windows; SPPS, Inc., Chicago, IL). Specificity, sensitivity, and predictive values for SR disease are also reported.

All flow cytometric analyses were conducted using software (FlowJo; Tree Star Software, San Carlos, CA). For the IL-2 inhibitor studies, cells exposed to a supratherapeutic concentration of tacrolimus (1 mg/mL) did not proliferate, and a marker set on this population was then applied to all other samples to determine their proportion of nonproliferated cells, as previously described.[10] Statistical comparisons were then calculated by Mann-Whitney test (GraphPad Prism ver. 4.00 for Windows; GraphPad Software, San Diego, CA).

RESULTS

Characteristics of Study Subjects

Of the 27 patients with sight-threatening intraocular inflammation, 14 had clinically SR disease (i.e., the threshold for reactivation during their first cycle of corticosteroid monotherapy was ≥10 mg prednisone daily), with a median age of 43 years (vs. 31 years for patients with SS uveitis; Table 1). The distribution of the sexes was similar in both groups, but there was a higher frequency of intermediate uveitis, classified in accordance with SUN guidance,[9] (perhaps reflecting the greater number of patients with associated neuroinflammatory conditions) in the SR cohort. Almost all patients had longstanding, chronic bilateral disease and all but one were white. None of those with SS disease had active uveitis by SUN criteria,[9] whereas only 78% of those with SR uveitis had inactive disease on enrollment. The proportion of patients with systemic disease, diagnosed on the basis of an appropriate subspecialist consult, was equivalent in both groups. Prednisone use was also equivalent at the time of enrollment, with a median dose of 8 mg daily in the SR group and 7.5 mg daily in the SS group. There was greater use of mycophenolate mofetil and methotrexate as additional immunosuppressants in patients with SR uveitis, although tacrolimus use was similar in both cohorts.

Clinical versus In Vitro Measures of Steroid Sensitivity

Of the 10 patients with SR disease in vitro, 9 also had clinically SR disease, and of the 17 with in vitro SS, 12 also had clinically SS uveitis (Table 2). This good level of agreement corresponds to a κ coefficient of 0.56 ($P = 0.002$). Specificity and sensitivity were 92% and 64%, respectively, giving this in vitro measure a positive predictive value of 90% and a negative predictive value of 71% for clinically defined disease. In a subgroup analysis of patients with isolated ocular inflammation ($n = 19$) and patients with associated systemic diagnoses ($n = 8$), the positive predictive value for SR disease was 83% and 100% respectively. CFSE profiles for individual patients are shown in Figure 2.

Dex versus IL-2 Inhibitor Suppression of $CD4^+$ Cell Proliferation

CFSE dilution in $CD4^+$ cells from four normal volunteers in the presence of increasing concentrations of Dex are shown in Figure 3A. All these individuals are defined as in vitro SS on the basis of the criteria set out in Figure 1, and although the Dex concentration at which maximum inhibition of proliferation is achieved varied, their CFSE profiles with supratherapeutic concentrations (10^{-6} M) of Dex was consistent. All had a persis-

TABLE 1. Characteristics of Patients

Characteristics	Clinical Classification	
	SR	SS
Patients, *n*	14	13
Median age at presentation, y (range)	43 (21–68)	31 (21–72)
Sex, % of patients (*n*)		
Male	28.6 (4)	38.5 (5)
Female	71.4 (10)	61.5 (8)
Race, % of patients (*n*)		
White	100 (14)	92.3 (12)
Black	0 (0)	7.7 (1)
Classification of uveitis, % of patients (*n*)		
Anterior	0 (0)	7.7 (1)
Intermediate	85.8 (12)	53.8 (7)
Posterior	7.1 (1)	15.4 (2)
Pan	7.1 (1)	23.1 (3)
Systemic diagnosis, % of patients (*n*)		
None	71.4 (10)	69.2 (9)
Behçet's disease	0 (0)	7.7 (1)
Sarcoidosis	0 (0)	15.4 (2)
Multiple sclerosis	14.4 (2)	0 (0)
Ankylosing spondylitis*	0 (0)	7.7 (1)
Other, nonspecific neuroinflammation	7.1 (1)	0 (0)
Other, psoriasis*	7.1 (1)	0 (0)
Bilateral disease, % of patients (*n*)	100 (14)	92.3 (12)
Right	0 (0)	7.7 (1)
Left	0 (0)	0 (0)
Uveitic activity, % of patients (*n*)†		
Active	21.4 (3)	0 (0)
Inactive	78.6 (11)	100 (13)
Systemic disease activity, % of patients (*n*)†		
N/A	71.4 (10)	69.2 (9)
Active	21.5 (3)	15.4 (2)
Inactive	7.1 (1)	15.4 (2)
Uveitis history		
Median disease duration prior to enrollment, y		
Since start of this episode‡	4.3	4.4
Since first presentation	5.1	4.4
Systemic treatment, *n* (median dose)†		
Prednisone	13 (8 mg/d)	10 (7.5 mg/d)
Mycophenolate mofetil	11 (2 mg/d)	4 (2.5 mg/d)
Tacrolimus	7 (4 mg/d)	9 (4 mg/d)
Methotrexate	2 (12.5 mg/wk)	0 (0 mg/wk)

* Patients with ankylosing spondylitis or psoriasis did not have associated inflammatory bowel disease

† At the time of study enrollment.

‡ This episode refers to the current period of inflammation. For patients with recurrent disease, it started when the disease reactivated at the end of the last period of remission. For patients with chronic disease, it started at first presentation.

tent, small CFSE^{low} subpopulation, where the maximum CFSE dilution was equivalent to positive control cultures (stimulated with CD3-CD28 in the absence of Dex or IL-2 inhibition). This finding was not an artifact of variable preculture CFSE staining, as undivided control cells exposed to 1 mg/mL tacrolimus had uniformly high fluorescence (Fig. 3B), suggesting that cells escaping Dex inhibition proliferate at the same rate and therefore undergo the same number of divisions as untreated cells. Conversely, the CFSE profile of cultures exposed to agents that inhibit the generation and action of IL-2 have a very different pattern of proliferation (Figs. 3B, 3C). They have a second CFSE peak closely adjacent to undivided CFSE^{hi} cells, and their maximum CFSE dilution reduces with increasing IL-2 inhibitor concentration, indicating that although anti–IL-2 agents may have less effect than Dex on the proportion of cells that go into cycle, they more uniformly reduce the number of divisions that each cell undergoes.

CD4$^+$ cell suppression with a combination of 10^{-8} M Dex and an anti–IL-2 agent results in greater inhibition of proliferation than exposure to either agent alone (Fig. 3D; $P = 0.03$). The only exception is tacrolimus, which achieves maximum suppression at therapeutic concentrations in the absence of Dex.

DISCUSSION

This study demonstrates the cross-specialty relevance of the SR T-cell phenotype by replicating the previous identification of SR CD4$^+$ cells in UC[10] in a cohort of patients with uveitis. It also adds credence to the concept of GC-driven SR cell selection and strengthens the argument that this subpopulation of effector T cells represents a novel target for therapeutic intervention. In addition, the high level of agreement in the masked comparison of clinical and in vitro classifications of steroid sensitivity suggests that the reported CFSE-based assay has potential clinical application as a predictive test of steroid responsiveness.

This study also addresses one of the weaknesses of the previous UC study,[10] in that the control subjects were those with clinically SS disease who were also taking systemic immunosuppressants, as opposed to steroid naïve normal volunteers. Hence, differences observed in the study population cannot be attributed to disparities in exogenous corticosteroid use. However, prior in vivo exposure of PBMCs to prednisone in the present uveitis cohort may reciprocally be a confounding factor. This is also the case with the other immunosuppressant treatments that were being taken by participants, but it is counterintuitive that the greater burden of second-line agents (in particular, mycophenolate mofetil and methotrexate) in the SR group would have contributed to the greater degree of CD4$^+$ cell proliferation in their cultures. Conversely, it is striking that the same SR CFSE profile observed in drug-free patients with SR UC has been replicated in this uveitis cohort despite their concomitant systemic immunosuppression. However, other potential biases, such as the slightly higher proportion of patients with intermediate uveitis and active ocular inflammation in the SR group (Table 1), cannot be resolved in the absence of a prospective, case-matched study in treatment-naïve patients. Differences in uveitis etiology (e.g., Behçet's versus HLA-B27–associated disease) and disease severity were also not accounted for.

Despite these limitations, the 90% positive predictive value of the CFSE-based assay used in this study highlights its potential clinical utility (Table 2), and although we acknowledge the caveats associated with subgroup analyses in small patient

TABLE 2. Agreement between Clinical and In Vitro Measures of Steroid Sensitivity

	Clinical SR	Clinical SS	Total
In vitro SR	9	1	10
In vitro SS	5	12	17
Total	14	13	27

κ coefficient = 0.56 ($P = 0.002$). Positive predictive value, 90%; negative predictive value, 71%.

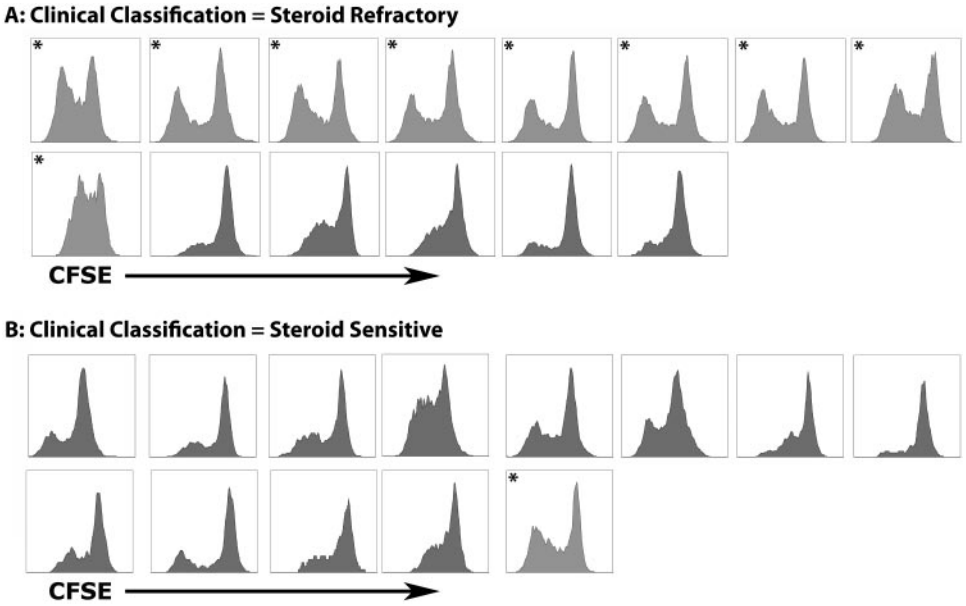

FIGURE 2. $CD4^+$ cell proliferation in PBMC cultures exposed to 10^{-6} M dexamethasone. (**A**) Patients with clinically SR ($n = 14$) or (**B**) clinically SS ($n = 13$) uveitis. *Light gray*: in vitro SR (*); *dark gray*: in vitro SS.

cohorts, the positive predictive value of 83% for isolated ocular inflammation suggests that this utility is maintained regardless of whether there is associated systemic disease. A priori identification of the SR phenotype would enable prompt introduction of alternative immunotherapies to circumvent the requirement for recurrent cycles of high-dose corticosteroid rescue after failed therapeutic taper. As such, this study reinforces that SR T cells are a strong candidate biomarker for SR disease, with genuine potential for translation for patient benefit. However, whether a patient's response to steroids is stable over time, or is influenced by disease activity, remains to be determined. Further investigation of the SR T-cell phenotype with the purpose of development of a predictive test for SR disease is therefore warranted.

Although the concept of an SR phenotype is relevant to a range of autoimmune diseases,[20] it does not manifest uniformly and clinical definitions of steroid nonresponsiveness vary across specialties. The SR threshold of 10 mg prednisone set in this study was informed by SUN guidance, but is lower than that used for SR UC,[7] SR asthma,[8] and SR rheumatoid arthritis.[21] Hence, the SR phenotype is unlikely to be absolute, and any functional T-cell assay used as a measure of steroid responsiveness will be accordingly disease specific. (In this study the culture conditions were preoptimized in patients with uveitis by using lower concentrations of CD3-CD28–coated beads [Dynabeads; Dynal, Ltd.] than that previously reported in patients with UC.) Although this emphasizes that clinical definitions of SR disease and their in vitro correlates are by their nature somewhat arbitrary, it is not at odds with the concept of an underlying common SR drive. The degrees of T-cell receptor stimulation and GC suppression that generate SR cells may vary, but once selected, they comprise a defined subpopulation that has now been identified in both SR uveitis and SR UC. As such, their further characterization has the potential to provide new insights into the mechanisms that underlie SR autoimmune inflammation, with the prospect of generating novel therapeutic strategies to augment or potentiate corticosteroid action. There is also potential to refine the clinical classification of SR disease by defining prednisone thresholds in terms of dose per unit body weight.

The accumulating evidence that IL-2 drives SR T cells[10,22] is supported by the greater suppression of proliferation observed in this study when Dex is used in combination with anti-CD25 mAbs, than when either agent is used alone (Fig. 3D). This finding may be explained by the complementary patterns of cell division seen in cultures of Dex and anti-IL-2–treated cells. These patterns suggest that although IL-2 inhibition is less effective at preventing cells from going into cycle, it uniformly reduces the extent of proliferation in those that do (Fig. 3B). In contrast, T cells escaping Dex suppression proliferated to the same degree as uninhibited control cells (Fig. 3A). This finding implies that GC suppression of T-cell proliferation is not entirely IL-2 dependent.[23] Consistent with this, IL-2 inhibitors and GCs are known to act at different points of the cell cycle, with the former preventing activation from G_0 to G_1,[24] and the latter inducing G_1 arrest.[25,26] SR cells that continue to divide in the face of Dex presumably therefore remain susceptible to specific IL-2 blockade.

It is striking that despite their disparate effects (Fig. 4), each of the anti-IL-2 agents tested induced the same pattern of T-cell inhibition (Fig. 3B), reinforcing the assumption of their shared mechanism of action. The similarity in the CFSE profiles from AG490- and CD25 mAb–treated cultures also singles out the JAK/STAT pathway as an alternative target for T-cell inhibition. Furthermore, the enhanced suppression of $CD4^+$ cell proliferation seen when AG490 was used in combination with Dex is in keeping with previous evidence that STAT-5 specifically inhibits GC action by preventing nuclear translocation of its cytosolic receptor.[11]

Although tacrolimus appears to be the most potent IL-2 inhibitor, its anti–T-cell effects may have been potentiated by the inhibition of tumor necrosis factor-α (TNF-α) secretion by monocyte-derived cells in the PBMC cultures.[27] This nonetheless reflects the in vivo tissue environment, and such an indirect, macrophage-mediated suppression of $CD4^+$ cell proliferation may also contribute to tacrolimus' clinically observed steroid-sparing success, as has recently been reported in a randomized controlled trial of tacrolimus versus cyclosporine for the treatment of noninfectious uveitis.[28] However, the clinical utility of the in vitro system reported in this study as a surrogate for patients' response to anti–IL-2 therapies remains untested. It should therefore not be assumed that the complete suppression of proliferation in $CD4^+$ cells from normal volunteers observed at therapeutic tacrolimus concentrations (10 ng/mL) in the absence of Dex (Figs. 3C, 3D) will extrapolate to uveitis cohorts, in

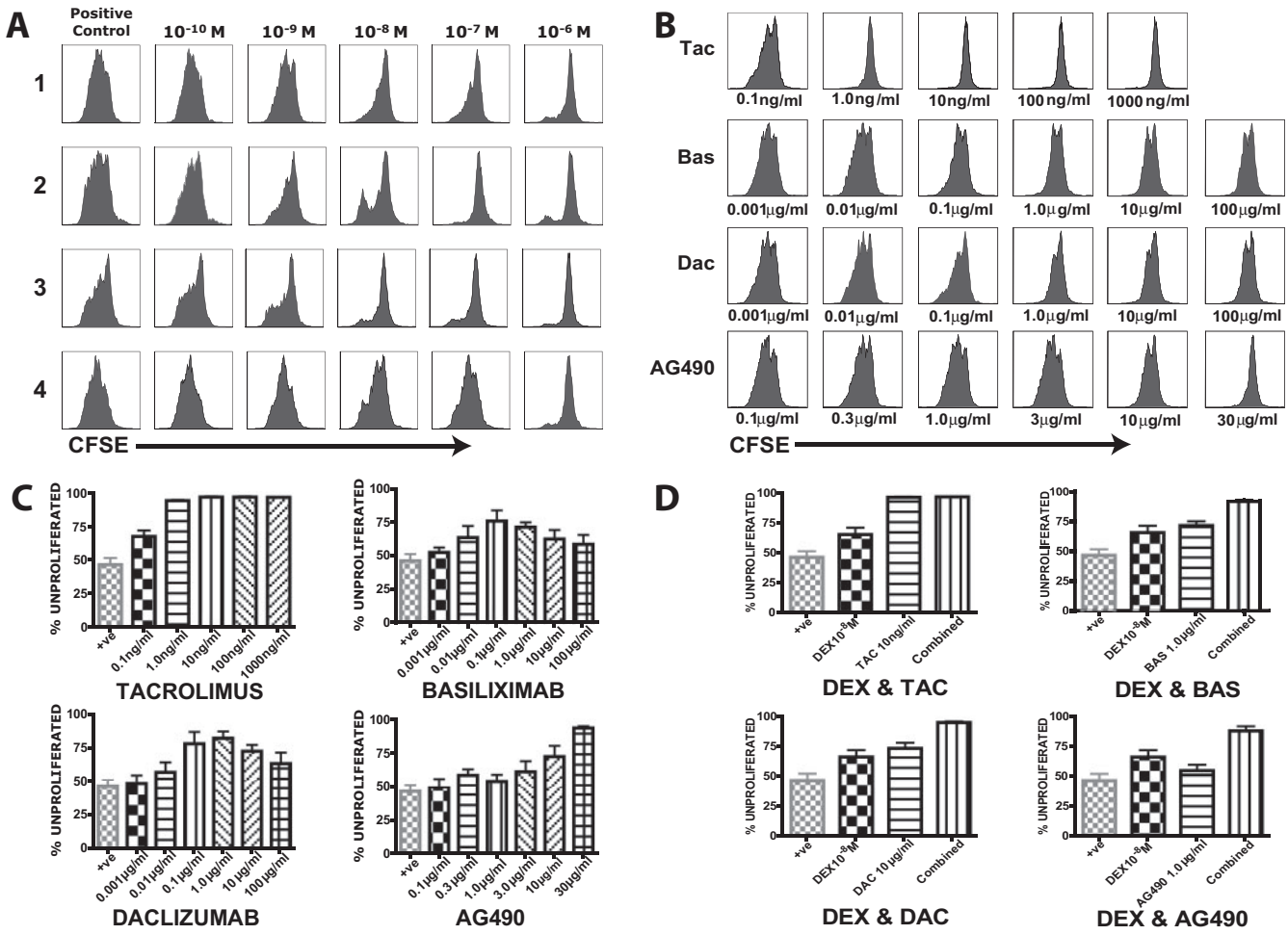

FIGURE 3. Dexamethasone (Dex) versus IL-2 inhibitor suppression of CD4$^+$ cell proliferation. (**A**) CD4$^+$ cell proliferation in PBMC cultures from four normal volunteers (1–4) in the presence of increasing concentrations of Dex. (**B**) Carboxyfluorescein succinimidyl ester (CFSE) dilution in CD4$^+$ cells from PBMC cultures exposed to increasing concentrations of IL-2 inhibitors, each of which targets a different point in the generation and action of IL-2 (see Fig. 4). The graphs show results for one donor who was representative of four normal volunteers. (**C**) Suppression of CD4$^+$ cell proliferation by each IL-2 inhibitor, quantified as the proportion of undivided cells ($n = 4$). (**D**) Combined Dex and IL-2 inhibitor suppression of CD4$^+$ cell proliferation (n=4). Tacrolimus (Tac), basiliximab (Bas), and daclizumab (Dac) were used at their therapeutic serum concentrations. As AG490 is not used clinically, a dose of 1.0 μg/mL was arbitrarily chosen. +ve, positive control.

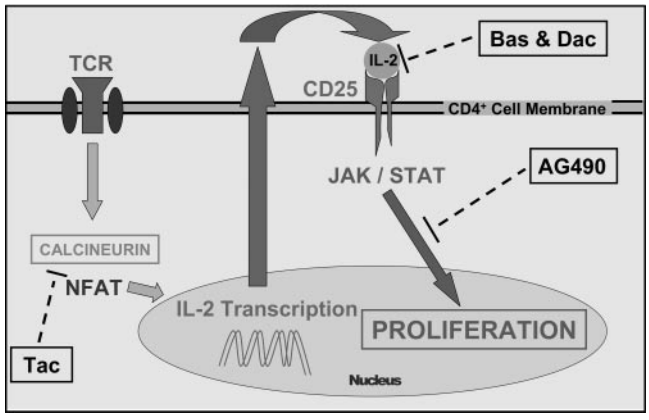

FIGURE 4. Mechanisms of IL-2 inhibitor action. Tacrolimus (Tac) inhibits dephosphorylation of nuclear factor of activated T cells (NFAT) by calcineurin, thus preventing NFAT's translocation to the nucleus where it drives IL-2 transcription. Basiliximab (Bas) and daclizumab (Dac) are, respectively, chimeric and humanized monoclonal antibodies to the α-subunit of the IL-2 receptor (CD25). AG490 inhibits the JAK/STAT pathway downstream of CD25. IL-2 is the canonical growth factor for T-cell division and proliferation. *Dashed lines*: points of inhibition.

which up to 40% of tacrolimus-treated patients are unable to discontinue prednisone.[29]

In summary, this study has identified an SR subpopulation of CD4$^+$ cells which is prevalent in patients with SR uveitis and is analogous to the SR CD4$^+$ cells previously identified in patients with SR UC. This result reinforces the concept of a common steroid refractory phenotype which has potential cross-specialty importance in the treatment of autoimmune disease. There is also clear potential for using SR CD4$^+$ cells as biomarkers of SR disease. Furthermore, the different effects of anti–IL-2 agents and GCs on T-cell proliferation provide insight into the mechanisms by which calcineurin inhibitors and anti-CD25 mAbs exert their clinically observed steroid-sparing effects. Accordingly, the characterization of SR CD4$^+$ cells has the potential to identify novel therapeutic targets for intervention in SR disease, a prime candidate for which is JAK/STAT inhibition.

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