

Steroid Hormone Regulation of Cytokine Secretion by Proteolipid Protein-Specific CD4⁺ T Cell Clones Isolated from Multiple Sclerosis Patients and Normal Control Subjects¹

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Steroid hormones have long been known to modulate immune function, and recent studies indicate that one of the means by which they do so involves effects on the secretion of immunoregulatory cytokines. Our laboratory has found recently that estradiol (E2) selectively modifies cytokine secretion in proteolipid protein (PLP)-specific, CD4⁺ T cell clones isolated from patients with the demyelinating disease, multiple sclerosis, and from normal control subjects. The data suggest that E2 may play a role in regulating the balance between pro- and antiinflammatory conditions, especially at concentrations typical of pregnancy. To determine whether other pregnancy-associated steroid hormones are capable of similar activity, we expanded our testing to include estrone (E1), estriol (E3), progesterone, and dexamethasone. The results indicate that E1 and E3 enhance secretion of Ag- or anti-CD3-stimulated IL-10 and IFN- γ in dose-dependent fashion, almost identical to that of E2. The effect on IL-10 was more potent than occurred with IFN- γ . In addition, E1 and E3, like E2, had a biphasic effect on TNF- $\alpha\beta$ secretion, with low concentrations stimulatory, and high doses inhibitory. None of the estrogens influenced IL-4 or TGF- β secretion. Progesterone enhanced secretion of IL-4, without affecting any other tested cytokine. Finally, dexamethasone induced TGF- β secretion, but inhibited IFN- γ and TNF- $\alpha\beta$. This differential effect of steroid hormones on the secretion of cytokines by CD4⁺ human T cell clones is consistent with the possibility that, collectively, they promote antiinflammatory conditions at high concentrations typical of pregnancy. *The Journal of Immunology*, 1998, 161: 3365–3374.

Cytokines produced by activated CD4⁺ lymphocytes play an important role in the initiation and regulation of immune responses. It is clear that the pattern of cytokines produced upon exposure to Ag is an important determinant of the relative involvement of cytotoxic and inflammatory, or humoral and antiinflammatory activities throughout a particular response. Cytokines that promote cell-mediated immune responses such as delayed-type hypersensitivity include IL-2, IFN- γ , and TNF- β (lymphotoxin), while Ab production, especially IgE, is promoted by IL-4, IL-5, IL-6, IL-10, and/or IL-13. CD4⁺ T cells that secrete these two patterns were originally identified in murine T cell clones (TCCs) and designated Th1, for the inflammatory cytokine pattern, and Th2, for the Ab-promoting pattern (1–3). Similar CD4⁺ T cell subsets have been identified in human TCCs, although they show less restricted cytokine secretion patterns; i.e., IL-2, IL-6, IL-10, and IL-13 may be produced by either subset (3, 4). However, it is generally agreed that the hallmark of Th1- or Th2-like identity in both murine and human T cells consists of, respectively, the secretion of IFN- γ or IL-4 (3). A third subset,

Th0, secretes combinations of both Th1- and Th2-like cytokines, and a fourth, designated Th3, produces large quantities of the potent antiinflammatory cytokine, TGF- β (3, 5, 6), which may also be secreted by Th0, Th1, and/or Th2 cells.

Although it has become increasingly apparent that classification of CD4⁺ T cells as Th0, Th1, Th2, or Th3 does not adequately represent the existing heterogeneity in cytokine secretion patterns (3, 7), especially in human T cells, the Th1/Th2 classification has provided a useful framework for the study of natural and experimental allergic, infectious, or immune-mediated diseases that show Th1 and Th2 biases associated with specific manifestations of disease (8, 9). For example, CD4⁺ T cells isolated from patients with multiple sclerosis (MS)⁴ show predominance of Th1- or Th2-like character depending upon disease activity (10). MS is an inflammatory demyelinating disease of the central nervous system in which CD4⁺ T cells reactive against myelin proteins are thought to play a role in myelin damage (11, 12). The majority of myelin Ag-specific T cells isolated during active disease secrete the Th1-like cytokines IFN- γ and TNF- $\alpha\beta$ (10, 13, 14), while during remission, IL-4- and IL-10-producing Th2-like cells emerge (10). In addition, TCCs capable of secreting TGF- β are rarely isolated during acute attacks of MS, but are readily cultured from patients during remission (10), suggesting an antiinflammatory influence during this stage. TCCs cultured from healthy control subjects showed a Th0 pattern of cytokine secretion. The pathogenic potential of Th1-like cytokines in MS is illustrated by the observation that systemic administration of IFN- γ resulted in worsening of clinical symptoms (15). Similarly, CD4⁺ Th1 cells induce relapsing-remitting experimental allergic encephalomyelitis (EAE), an

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⁴ Abbreviations used in this paper: MS, multiple sclerosis; E1, estrone; E2, estradiol; E3, estriol; Dex, dexamethasone; EAE, experimental allergic encephalomyelitis; ERE, estrogen response element; PLP, proteolipid protein; Prog, progesterone; rh, recombinant human; RR, relapsing-remitting; TCC, T cell clone.

animal model of MS in which remissions are accompanied by increases in antiinflammatory, Th2-like cytokines, particularly IL-10 (11, 16–18). Moreover, inflammatory CNS infiltrates are surrounded by TGF- β and IL-4 immunoreactivity during spontaneous recovery, and in mice rendered tolerant to EAE induction by oral administration of myelin (18). Interestingly, Th1-like T cells and cytokines are also thought to play a role in the pathogenesis of rheumatoid arthritis (19) and autoimmune thyroid disease (20, 21). Collectively, these observations clearly indicate that the identification of factors involved in regulating cytokine secretion is crucial to our understanding of the pathogenesis of immune-mediated diseases and subsequent development of new treatment strategies.

An example of a physiologic, rather than a pathologic, state in which cytokine biases appear to occur is that of mammalian pregnancy (22, 23). Evidence has long accumulated to indicate that pregnancy is associated with enhanced humoral and reduced cellular immune activity, consistent with a bias for Th2-like cytokines (22–24). It has been hypothesized that interactions at the maternal-fetal interface, including cytokine secretion, are responsible for promoting an intrauterine Th2 environment that impacts systemic immune function (22). However, steroid hormones, which are secreted in large quantities during pregnancy, are clearly capable of regulating cytokine synthesis in a variety of cell types (25–27). Thus, it is possible that they may also play a role in regulating the balance between Th1- and Th2-like activity. For instance, glucocorticoids inhibit IL-2 and IFN- γ (25, 28), induce TGF- β secretion (29), and decrease IL-2R expression in T cells (30). The adrenal androgen, dihydroepiandrosterone and 1, 25 (OH)₂ vitamin D3 selectively evoke, respectively, Th1- or Th2-like cytokine secretion patterns in mice (25). Estradiol (E2) has also been reported to enhance the activity of the IFN- γ promoter, which contains sequences resembling the consensus estrogen response enhancer (ERE) (31). Finally, we have found recently that E2 enhances Ag- and anti-CD3-stimulated IL-10, and to a lesser extent, IFN- γ secretion in myelin Ag-specific TCCs from MS patients and normal control subjects (32). The effect was dose dependent, and occurred at pregnancy-associated concentrations regardless of Th subset identity and disease status. The possibility that E2 supports a bias for Th2-like, or antiinflammatory conditions during pregnancy was further supported by the ability of high doses to inhibit secretion of TNF- $\alpha\beta$ in these clones.

In this communication, we have expanded the testing of steroid effects on cytokine secretion in human CD4⁺ TCCs to include estrone (E1), estriol (E3), progesterone (Prog), and glucocorticoids, all of which are elevated significantly during pregnancy. The data indicate that they act selectively to affect the secretion of individual cytokines regardless of Th subset identity. In addition, the data are consistent with the possibility that these hormones act collectively to promote antiinflammatory, or Th2-like activity at pregnancy-associated concentrations. Finally, the data have implications for our understanding of the mechanisms by which cytokine gene expression may be regulated in general.

Materials and Methods

Subjects

Ten patients with clinically definite MS (six patients with relapsing-remitting MS, designated RR, and four patients with progressive MS, designated CP), and two healthy control subjects, designated NC, were the source of the TCCs included in this study. Details about these patients, including disease characteristics, have been published previously (10, 32–35). Subjects BP, LW, RK, RP, RR, RSP, SS, CP, and ML are female; male subjects include JC, PK, and MK. For the patients with RR disease, clones were isolated during successive relapses, or acute attacks, and remissions of clinical symptoms. None of the patients had received steroids or immunosuppressive drugs for at least 3 mo before blood sampling. If the patient

was in the midst of an acute attack, blood was drawn before administration of i.v. steroids. The research project was reviewed and approved by University of Southern California Institutional Review Board (Los Angeles).

Establishment of proteolipid protein-specific TCCs

CD4⁺ TCCs specific for antigenic peptides derived from the myelin proteolipid protein (PLP) were isolated from PBMC collected from MS patients and normal control subjects, as previously described (33). Briefly, 5×10^6 PBMC were cultured in the presence of 10 to 25 $\mu\text{g/ml}$ of synthetic PLP 104–117 or PLP 142–153 peptides. Five to seven days later, cells were expanded in fresh medium containing 50 U/ml of rhIL-2 (a generous gift from Cetus, Emeryville, CA) for an additional 7 days. Cultures were then submitted to alternating cycles of weekly restimulation and expansion until Ag specificity was evident in proliferation assays. Restimulation was accomplished in the presence of antigenic PLP peptide and autologous, irradiated (3000 rad), unfractionated PBMC as APCs, while expansion occurred in the presence of 50 U/ml rhIL-2. PLP peptide-specific cells were then cloned by limiting dilution and maintained in serum-free medium (Ex-Vivo 20; BioWhittaker, Walkersville, MD) by restimulation/expansion cycles that alternated every 10 to 14 days. To avoid possible influences of in vivo disease activity on APC function, the TCCs from RR MS patients were developed, cloned, and tested using autologous APCs from the same stage of disease from which the TCCs were isolated. That is, TCCs isolated during acute attacks of disease were always cultured with autologous APCs collected during acute attacks, and those isolated during remission were cultured in the presence of autologous APCs collected during remission. All TCCs exhibited a CD3⁺CD4⁺CD8⁻TCR $\alpha\beta$ ⁺ cell surface phenotype, identified by immunofluorescence staining and FACS analysis. True clonality was indicated by homogeneous staining patterns obtained with anti-TCR V β mAbs (33).

Assignment of Th identity and selection of clones for study

The 38 PLP-TCCs included in this study exhibited stable cytokine secretion patterns identifiable as Th0-, Th1-, or Th2-like in response to the appropriate PLP peptide and APCs, or to stimulation with anti-CD3 mAb. All of these clones are listed in Table I, along with the cytokines they secrete and which cytokines were tested for sensitivity to E1, E3, Prog, and Dex. Primary criteria for assignment of T cell subset identity were as follows: 1) Th1-like identity was assigned if a clone secreted IFN- γ , but not IL-4; 2) Th2-like identity was assigned if IL-4, but not IFN- γ , was secreted; and 3) Th0-like identity was assigned if both IFN- γ and IL-4 were secreted. Th0, Th1, and Th2 clones identified in this fashion may also produce IL-10 and/or TGF- β , the secretion of which is not restricted to Th subset in human cells. None of the Th2-like clones secreted TNF- $\alpha\beta$. All were tested at least three times under similar conditions to confirm the stability of these cytokine secretion patterns (10, 32). Overall, 22 Th0-like, 12 Th1-like, and 4 Th2-like PLP-TCCs were included in this study, which was initially designed to determine whether these clones are sensitive to E1, E3, Prog, and/or Dex. The clones were selected from a panel of more than 150 PLP-TCCs to include representatives from each Th subset, from each stage of disease in the MS patients, and from healthy control subjects. In addition, clones were included that had been tested previously for sensitivity to E2 (32).

Stimulation of TCCs for cytokine production

PLP peptide-specific TCCs were tested for cytokine production 7 to 10 days after their last stimulation with feeder cells. For peptide Ag-specific cytokine secretion, PLP-TCCs were cultured in 96-well plates at a density of 5×10^4 cells/well in the presence of 5×10^3 adherent irradiated autologous PBMC as a source of APCs (referred to subsequently as Ag/APCs), 10 to 25 $\mu\text{g/ml}$ of the appropriate PLP peptide, and various concentrations of E1, E3, Prog, or Dex (Sigma, St. Louis, MO). Steroid hormones were dissolved in absolute ethanol at a stock concentration of 0.01 M and further diluted in serum-free medium to the tested concentrations. All experiments were conducted in serum-free medium. Supernatants were collected at 36 h (for IL-4 detection) or 72 h (for all other cytokines) and stored at -70°C until testing. For nonspecific stimulation, PLP-TCCs (5×10^4 cells/well) were cultured in the presence of 1 $\mu\text{g/ml}$ of immobilized anti-CD3 mAb (OKT3; American Type Culture Collection (ATCC), Manassas, VA) in the presence or absence of steroids. Irradiated PBMC (APCs) were not included under these conditions. All clones were tested for sensitivity to each steroid under these two stimulation conditions; thus, the minimum number of times each clone was tested was twice. However, most clones were tested three to five times in the presence of the estrogens and two to four times in the presence of Prog or Dex. Although cells stimulated with anti-CD3 tended to secrete higher levels of cytokine than

Table I. Summary of the characteristics of all the CD4⁺ PLP peptide-specific T cell clones used in this study (n = 38), including Th subset assignment, the pattern of cytokines secreted by each, and which cytokines were tested for sensitivity to the indicated steroid hormones

T Cell Clone ^a	Donor ^b	Th Subset ^c	Estrone ^d	Estriol	Prog	Dex
BPAA142-3	MS AA	Th0: 4, 10, IFN	4, 10	4, 10	4, 10	
BPRem104-6	MS Rem	Th0: 4, IFN, TNF, TGF				TGF
BPRem142-4	MS Rem	Th0: All	TGF	TGF	4, TGF	TGF
BPRem142-6	MS Rem	Th0: All				TGF
LWAA104-1	MS AA	Th0: 4, 10, IFN, TNF	4	4	4	IFN
LWAA104-2	MS AA	Th1: IFN, TNF	IFN, TNF	IFN, TNF	IFN, TNF	IFN
LWAA142-2	MS AA	Th0: 4, 10, IFN, TNF	10	10	10	TNF
LWRem104-2	MS Rem	Th2: 4, 10, TGF	TGF	TGF	4, TGF	TGF
LWRem104-4	MS Rem	Th0: 4, 10, IFN, TNF			10	
LWRem104-5	MS Rem	Th2: 4, 10, TGF	TGF	TGF	TGF	TGF
RKAA104-1	MS AA	Th1: 10, IFN, TNF	IFN	IFN	IFN	IFN
RKAA104-4	MS AA	Th1: 10, IFN, TNF	10, IFN, TNF	10, IFN, TNF	10, IFN, TNF	TNF
RSPAA104-1	MS AA	Th0: 4, 10, IFN	4	4	4	
RSPRem104-3	MS Rem	Th2: 4, TGF			TGF	TGF
RSPRem142-2	MS Rem	Th0: 4, 10, IFN	4	4	4, 10	
SSAA104-1	MS AA	Th1: IFN, TNF				IFN
SSAA104-3	MS AA	Th0: 4, 10, IFN, TNF	4, 10, TNF	4, 10, TNF	4, 10, TNF	
SSRem104-1	MS Rem	Th0: 4, 10, IFN, TNF	IFN	IFN	4, 10, IFN	
SSRem104-3	MS Rem	Th1: IFN, TNF, TGF	TNF, TGF	TNF, TGF	TNF, TGF	TGF
SSRem104-4	MS Rem	Th1: IFN, TNF, TGF	TGF	TGF	TGF	TGF
SSRem104-5	MS Rem	Th2: 4, 10	10	10	10	
CP6	MS CP	Th0: All				4
CP8	MS CP	Th0: All				4, 10
MK104-1	MS CP	Th1: IFN, TNF				IFN
MK104-2	MS CP	Th1: IFN, TNF				IFN
ML104-1	MS CP	Th1: IFN, TNF				IFN
PK104-3	MS CP	Th1: IFN, TNF				IFN, TNF
PK104-4	MS CP	Th1: IFN, TNF				IFN, TNF
PK104-6	MS CP	Th1: IFN, TNF				IFN, TNF
RR6	MS Rem	Th0: 4, 10, IFN				4, 10
RR7	MS Rem	Th0: All				4, 10
RR8	MS Rem	Th0: All				4, 10
RR9	MS Rem	Th0: 4, 10, IFN, TNF				4, 10
JC104-2	NC	Th0: All	All	All	All	
JC104-3	NC	Th0: All	10, IFN, TNF	10, IFN, TNF	All	
RP104-1	NC	Th0: All	4, 10, IFN, TGF	4, 10, IFN, TGF	4, 10, IFN, TGF	
RP104-3	NC	Th0: 4, 10, IFN, TNF	4, IFN	4, IFN	4, 10, IFN	
RP104-4	NC	Th0: All	TGF	TGF	TGF	

^a T cell clones are named for the individual donor, disease status, and PLP peptide specificity (either PLP 104–117, designated 104, or PLP 142–153, designated 142).

^b Donors were relapsing-remitting MS patients in acute attack (AA) or remission (Rem), MS patients with chronic progressive disease (CP), or normal control subjects (NC).

^c The subset identity and cytokines secreted are identified for each individual clone. Criteria for assignment of Th subset identity are presented in *Materials and Methods*. Cytokines are designated in the table as follows: 4, IL-4; 10, IL-10; IFN, IFN- γ ; TNF, TNF- α ; TGF, TGF- β ; All, indicates that all five cytokines were secreted. The number of Th0, Th1, and Th2 clones included in this study was, respectively, 22, 12, and 4.

^d Cytokines tested in the presence of each steroid hormone are indicated for each individual T cell clone (does not indicate whether or not cytokine secretion changed in the presence of hormone).

when stimulated with peptide Ag/APCs, there was no difference in the pattern of cytokine secretion stimulated under these two conditions.

Experiments were also conducted to address the possibility that the irradiated adherent autologous PBMCs used as APCs in these studies play a primary role in the hormone influence on cytokine secretion by the PLP-TCCs. For this purpose, APCs were cultured in the presence and absence of PLP-TCCs (n = 18) and PLP peptide, and supernatants were collected as described above. Each clone was also stimulated by anti-CD3 in the absence of Ag/APCs.

Additional controls included viability studies using trypan blue dye exclusion, the addition of ethanol diluted to concentrations equivalent to those used for hormone dilutions, and serum-free medium without phenol red. The latter control was included to rule out the estrogen-like activity of phenol red (36).

Measurement of cytokine secretion

The concentrations of IL-4, IL-10, IFN- γ , TNF- α , and TGF- β in the culture supernatants were determined as previously described (10, 32). Briefly, IL-4, IL-10, and IFN- γ were measured by capture ELISAs developed in our laboratories using mAbs purchased from PharMingen (San Diego, CA). Standard curves were generated using recombinant human cytokines, purchased from PharMingen (IL-4 and IL-10) or Genzyme (Cambridge, MA; IFN- γ). Detection sensitivity was 45 pg/ml for IL-4, 50 pg/ml for IL-10, and 100 pg/ml for IFN- γ .

TNF- α activity was quantified as a measure of cytotoxicity using actinomycin D-treated L929 cells as targets, with vital dye uptake as a spectrophotometric end point (10, 32). Data are expressed as U/ml, which represents the reciprocal of the highest dilution resulting in 50% lysis, multiplied by the sample dilution factor.

Active TGF- β was determined as a measure of proliferation inhibition using the mink lung epithelial cell line Mv1Lu (ATCC CCL-64), as previously described (10, 32). A standard curve (TGF- β concentrations versus percentage of inhibition) for each assay was constructed using rhTGF- β (R&D Systems, Minneapolis, MN). The sensitivity of the assay was 100 pg/ml. CCL-64 and L-929 cells were not affected by the direct addition of Ag plus APCs or by any tested concentration of E1, E3, Prog, or Dex.

Statistical analysis

The Mann-Whitney U test was applied to evaluate differences in the effect of E1 and E3 on IL-10, IFN- γ , and TNF- α secretion. The data were expressed as percentage of change in cytokine secretion relative to baseline cytokine secretion in the absence of hormone. *p* values \leq 0.05 were considered significant.

Results

Recent studies in our laboratories have revealed that IL-10, IFN- γ , and TNF- α secretion by PLP-TCCs are sensitive to the presence

Table II. Summary of steroid hormone effects on cytokine secretion by human Ag specific T cell clones^a

Hormone	IL-4	IL-10	TGF- β	IFN- γ	TNF- $\alpha\beta$
Estrone (E1) ^b	0/8	6/8 (75%)	0/8	6/8 (75%)	5/6 (83%)
Estriol (E3) ^b	0/8	6/8 (75%)	0/8	6/8 (75%)	5/6 (83%)
Progesterone ^c	12/12 (100%)	0/12	0/8	0/8	0/6
Dexamethasone ^d	0/6	0/5	8/8 (100%)	10/10 (100%)	4/5 (80%)

^a T cell clones ($n = 38$) were isolated from patients with MS during acute attacks or clinical remissions and from normal control subjects. All are CD4⁺, specific for PLP peptide 104–117 or 142–153, and secrete cytokine secretion patterns typical of Th0, Th1, or Th2 cells. Cytokines were measured in supernatants of Ag- or anti-CD3-stimulated clones by ELISA (IL-4, IL-10, IFN- γ) or bioassay (TNF- $\alpha\beta$ and active form of TGF- β). Data are expressed as the number of hormone-responsive clones per number of clones tested; percent responsive is indicated in parentheses.

^b The effect of E1 and E3 on IL-10 and IFN- γ was an enhancement. For TNF- $\alpha\beta$, the effect was biphasic; i.e., low concentrations enhanced, while high concentrations inhibited its secretion.

^c The effect of progesterone on IL-4 secretion was an enhancement.

^d The dexamethasone effect on IFN- γ and TNF- $\alpha\beta$ was one of inhibition, while TGF- β secretion was enhanced.

of E2, especially at concentrations typical of circulating levels during pregnancy (32). For IL-10, Ag- and anti-CD3-stimulated secretion was enhanced in dose-dependent fashion, with the greatest enhancement magnitude occurring at the highest tested E2 dose. A similar pattern of enhancement occurred for IFN- γ secretion, although it was significantly less robust, and peaked at lower E2 concentrations. By contrast, the E2 effect on TNF- $\alpha\beta$ secretion was biphasic with respect to E2 dose; low concentrations, typical of circulating doses during the normal menstrual cycle, were stimulatory, while pregnancy-associated concentrations were inhibitory. E2 had no effect on IL-4 or TGF- β secretion. The effects were cytokine specific, since they occurred independent of Th subset identity and disease status of the donor. These data suggest that E2 is capable of contributing to an antiinflammatory, or Th2-like bias that has been proposed to occur during pregnancy (22–24). To determine whether other steroid hormones that are elevated during pregnancy share this capability, E1, E3, Prog, and Dex were added to cultures of 38 Ag- or anti-CD3-stimulated PLP-TCCs, and IL-4, IL-10, IFN- γ , TNF- $\alpha\beta$, and TGF- β were measured in culture supernatants. The data are described in detail for each individual hormone, as follows.

The effect of E1 and E3 on cytokine secretion by CD4⁺ PLP peptide-specific TCCs

As summarized in Table II, eight PLP-TCCs were tested for sensitivity to the effects of E1 and E3 on IL-4, IL-10, IFN- γ , and TGF- β , while six clones were tested for TNF- $\alpha\beta$ sensitivity. None of the clones showed changes in IL-4 or TGF- β secretion upon stimulation by Ag/APCs or anti-CD3 (data not shown), as previously published for E2 (32). Six of the eight clones (75%) showed enhancement of IL-10 and IFN- γ secretion in the presence of E1 and E3. IL-10 enhancement was dose dependent and first evident at 5,000 to 10,000 pg/ml, reaching a maximum at the pharmacologic dose of 100,000 pg/ml (Fig. 1, *a*, *b*, and *g*; Table III). At this dose, enhancement varied with the individual clone and experiment from approximately 150 to 650%. The magnitude of enhancement at each dose is most clearly presented by expressing the data as percentage of change, as illustrated for E1 in Figure 1*b*. Percentage of enhancement data for E3 effects on IL-10 secretion are also presented in Table III for representative clones stimulated by Ag/APC and anti-CD3. Physiologic concentrations of E1 and E3 are roughly equivalent to those of E2, which circulates at 10 to 1,000 pg/ml during the normal menstrual cycle, and up to 35,000 pg/ml during pregnancy. Pharmacologic ranges can be considered to be greater than 35,000 pg/ml.

As with IL-10, IFN- γ secretion by CD4⁺ Ag-specific TCCs was substantially increased in response to E1 and E3 during stimulation with cognate peptide Ag/APCs (Fig. 1, *c*, *d*, and *h*, and Table III)

or anti-CD3 mAb (Table III). Also in accord with the IL-10 results, the effect was dose dependent. However, enhancement was observed at lower E1 and E3 doses than for IL-10 (1,000 pg/ml vs 10,000 pg/ml), and peaked in all but one clone at 5,000 pg/ml. Maximum enhancement varied from 50 to 300% in most clones (for example, see Fig. 1*d* for percentage of enhancement in the presence of E1, and Table III for E3).

TNF- $\alpha\beta$ secretion by CD4⁺ TCCs in the presence of E1 and E3 showed a more complex secretion pattern than that of IL-10 and IFN- γ , and was identical to that previously observed with E2 (32). Thus, low concentrations of both E1 and E3 enhanced Ag/APC or anti-CD3-stimulated TNF- $\alpha\beta$ secretion, while high concentrations were inhibitory (Fig. 1, *e*, *f*, and *i*). Maximal TNF- $\alpha\beta$ release (70–500%) often occurred at 5,000 pg/ml, but could occur at 1,000 or 10,000 pg/ml, depending upon the individual clone and the experiment. Concentrations greater than 10,000 pg/ml significantly inhibited TNF- $\alpha\beta$ release, with maximal inhibition (85%) at 50 to 100,000 pg/ml. Eighty-three percent (5 of 6) of the PLP peptide-specific TCCs tested showed this pattern of responsiveness (Table II).

The suppressive effects of E1 and E3 on TNF- $\alpha\beta$ were not due to cell death, since viability, measured as trypan blue dye exclusion, was higher than 95% at any tested dose (data not shown). Furthermore, no statistically significant differences in IL-10, IFN- γ , and TNF- $\alpha\beta$ secretion were observed between E1 and E3. In addition, the estrogen effects were the same whether or not phenol red was present in the medium (data not shown).

Prog increases IL-4 secretion by PLP peptide-specific TCCs

As with the estrogens, Prog was tested in concentrations equivalent to those found in the peripheral circulation during the normal menstrual cycle (0.1–30 ng/ml), during pregnancy (10–400 ng/ml), as well as pharmacologic doses (12–35 ng/ml) (33). Figure 2 and Table III show that Prog clearly increases the capacity of activated PLP-TCCs to produce IL-4 in culture, and in fact, all tested TCCs (12 of 12; Table II) showed a reproducible susceptibility to this effect. In addition, the enhancement was dose dependent and clearly evident at concentrations higher than 10 ng/ml, with maximal effects at the highest concentration tested (1000 ng/ml). However, significant enhancement (>50%) could be observed at doses as low as 0.1 to 1 ng/ml (see Table III). Maximum enhancement varied with individual PLP-TCC from 152 to 2400% at 1000 ng/ml, most clearly demonstrated in Figure 2*b* and Table III. Enhancement occurred upon stimulation by either Ag/APCs (Fig. 2 and Table III) or anti-CD3 (Table III). Prog had no effect on the secretion of IL-10, IFN- γ , TNF- $\alpha\beta$, or TGF- β , at any of the concentrations tested (data not shown).

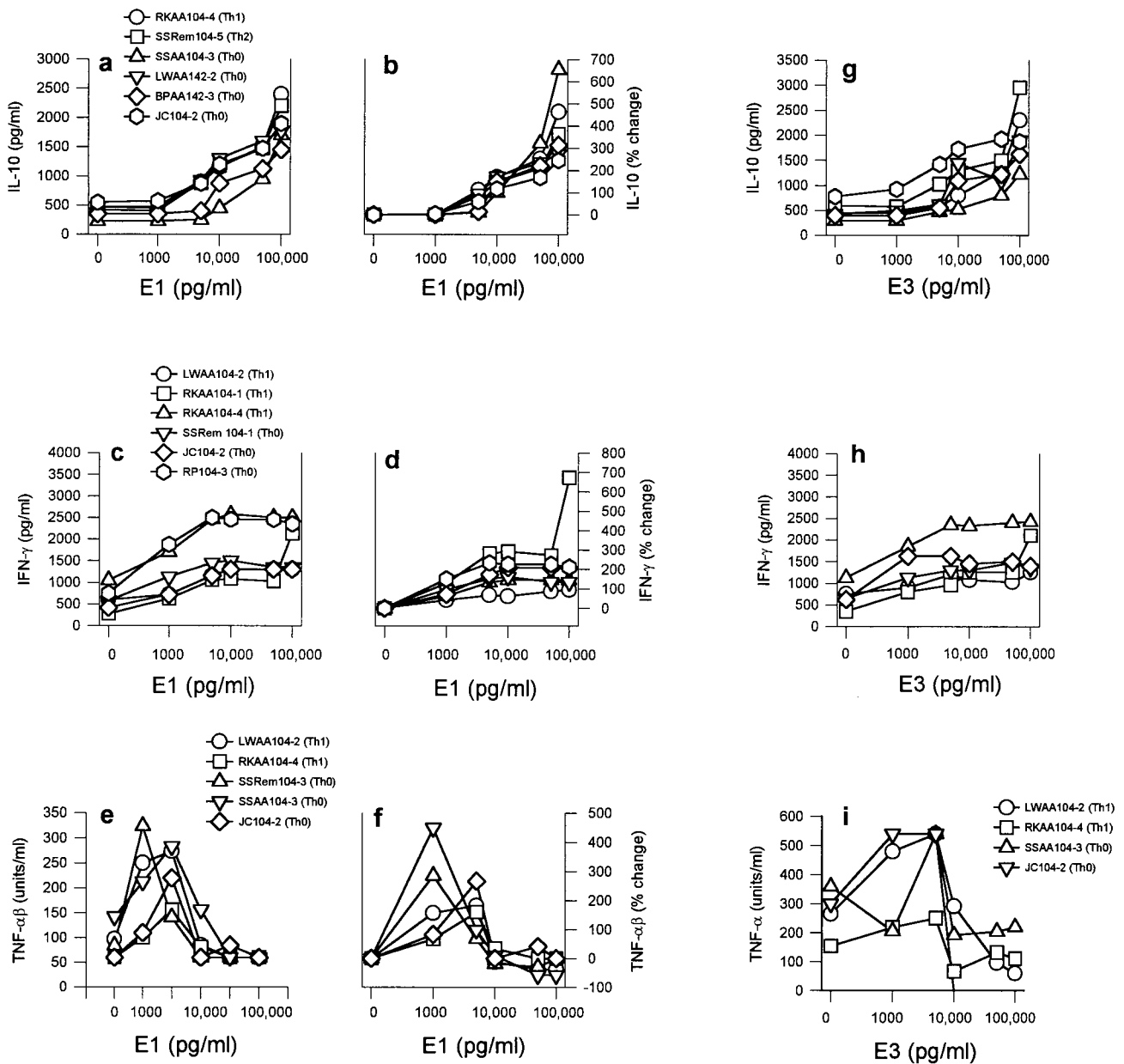


FIGURE 1. The effect of E1 and E3 on IL-10, IFN- γ , and TNF- $\alpha\beta$ secretion by PLP-TCCs. PLP-TCCs were isolated from MS patients and healthy blood donors. They were stimulated by cognate PLP peptide (PLP 104–117 or PLP 142–153) in the presence of irradiated autologous adherent PBMC as a source of APCs (Ag/APC), and various concentrations of E1 (a–f) or E3 (g–i). Supernatants were collected 72 h later and assayed for IL-10 (a, b, and g), IFN- γ (c, d, and h), and TNF- $\alpha\beta$ (e, f, and i), as described in *Materials and Methods*. The values are expressed as cytokine concentration (a, c, and e) or percentage of change in cytokine secretion (b, d, and f) for the same data as a function of steroid dose. Each symbol represents cytokine secretion by an individual clone. The data are representative of experiments repeated at least twice using the same PLP-TCCs stimulated by Ag/APC or immobilized anti-CD3 mAb. Six of eight PLP-TCCs (75%) showed enhancement of IL-10 and IFN- γ in the presence of E1 or E3, while five of six clones (four of which are illustrated) showed a dose-dependent biphasic pattern of TNF- $\alpha\beta$ secretion, in which low doses were stimulatory and high doses inhibitory. Maximum enhancement of IL-10 occurred in all responsive clones at 100,000 pg/ml of E1 and E3. For IFN- γ , enhancement was maximum at lower E1 and E3 doses, and was less robust. No significant differences were observed between the effects of E1 and E3. The subset identities are indicated in parentheses. Unresponsive clones are not included in the figures.

Dex inhibits IFN- γ and TNF- $\alpha\beta$ secretion and induces the release of TGF- β by PLP-TCCs

PLP peptide-specific TCCs from RR MS patients and NC donors were exposed to a broad range of Dex doses (10^{-9} to 10^{-4} mol/L) that are equivalent to serum concentrations of the naturally occurring corticosteroid, cortisol (0.01–1000 $\mu\text{g/ml}$). Panels a and b in Figure 3, and Table III clearly show that Dex significantly reduces the capacity of PLP-TCCs to produce IFN- γ in response to either Ag/APC or anti-CD3. This inhibitory influence was evident at con-

centrations ranging from 10^{-4} to 10^{-7} mol/L, with maximal inhibition (75–90%) at the highest concentration tested (most clearly observed in Fig. 3b). These effects were observed in all tested TCCs (10 of 10), which showed remarkable similarity in Dex responsiveness.

The effect of Dex treatment on TNF- $\alpha\beta$ release is presented in Figure 3, c and d, and in Table III. Compared with basal secretion in the absence of steroid, Dex-treated cells secreted significantly less TNF- $\alpha\beta$, especially at concentrations higher than 10^{-7} mol/L.

Table III. Steroid hormone effects on cytokine secretion by PLP-TCCs stimulated in the presence of anti-CD3, Ag-presenting cells, and Ag (designated TCC/Ag/APC) or Ag-presenting cells and Ag in the absence of PLP-TCCs (designated Ag/APC)^a

Cytokine	T Cell Clone	Stimulus	Hormone	Hormone Dose/Cytokine Concentration ^b						
				0	0.1	1	10	100	1,000	
IL-4	BPAA142-3	anti-CD3	Prog	766	1,199 (57) ^c	1,665 (117)	3,363 (339)	3,963 (417)	4,662 (509)	
		TCC/Ag/APC		499	499	1,299 (160)	2,897 (481)	4,129 (727)	4,629 (828)	
		Ag/APC		0	0	0	0	0	0	
	SSAA104-3	anti-CD3		433	300 (-31)	599 (38)	1,299 (200)	2,497 (477)	4,063 (838)	
		TCC/Ag/APC		300	300	433 (44)	800 (167)	2,497 (732)	3,430 (1043)	
		Ag/APC		0	0	0	0	0	0	
IL-10	RCAA104-4	anti-CD3	E3	0	1,000	5,000	10,000	50,000	100,000	
		TCC/Ag/APC		425	450 (6)	625 (47)	925 (118)	1,225 (172)	2,275 (405)	
		Ag/APC		450	450	700 (56)	750 (67)	1,125 (150)	2,025 (350)	
	LWAA142-2	anti-CD3		375	375	750 (100)	1,550 (313)	1,900 (407)	1,900 (407)	
		TCC/Ag/APC		350	400 (14)	750 (114)	1,500 (329)	1,750 (400)	2,250 (542)	
		Ag/APC		0	0	0	0	0	0	
	IFN-γ	JC104-2		anti-CD3	0	1,000	5,000	10,000	50,000	100,000
				TCC/Ag/APC	925	1,650 (78)	1,950 (111)	2,025 (119)	2,075 (124)	2,050 (122)
				Ag/APC	725	1,450 (100)	1,700 (134)	1,950 (169)	1,900 (162)	1,825 (152)
		RCAA104-1		anti-CD3	75	75	75	75	50	75
				TCC/Ag/APC	625	1,100 (76)	1,425 (128)	1,550 (148)	1,500 (140)	1,550 (148)
				Ag/APC	400	875 (119)	1,125 (181)	1,200 (200)	1,025 (156)	1,125 (181)
IFN-γ	SSAA104-1	anti-CD3	Dex	0	10⁻⁴	10⁻⁵	10⁻⁶	10⁻⁷	10⁻⁸	
		TCC/Ag/APC	1,225	275 (-78)	500 (-59)	775 (-37)	900 (-27)	1,050 (-14)		
		Ag/APC	1,050	200 (-81)	400 (-62)	675 (-36)	875 (-17)	975 (-7)		
	MK104-1	anti-CD3	75	75	100 (33)	125 (67)	100 (33)	125 (67)		
		TCC/Ag/APC	2,975	350 (-88)	925 (-69)	1,550 (-52)	2,050 (-31)	2,550 (-14)		
		Ag/APC	2,400	250 (-90)	550 (-77)	1,150 (-52)	1,375 (-43)	2,175 (-9)		
TNF-$\alpha\beta$	JC104-2	anti-CD3	E3	0	1,000	5,000	10,000	50,000	100,000	
		Ag/APC	376	540 (44)	458 (22)	150 (60)	108 (-71)	76 (-80)		
		Ag/APC	<20	<20	<20	<20	<20	<20		
	SSAA104-3	anti-CD3	183	513 (180)	166 (-9)	127 (-30)	123 (-33)	331 (81)		
		TCC/Ag/APC	<20	<20	<20	<20	<20	<20		
		Ag/APC	<20	<20	<20	<20	<20	<20		
TNF-$\alpha\beta$	PK104-3	anti-CD3	Dex	0	10⁻⁴	10⁻⁵	10⁻⁶	10⁻⁷	10⁻⁸	
		Ag/APC	235	175 (-26)	189 (-20)	268 (14)	172 (-27)	209 (-11)		
		Ag/APC	<20	<20	<20	<20	<20	<20		
	PK104-4	anti-CD3	288	161 (-44)	148 (-49)	140 (-51)	205 (-29)	259 (-10)		
		TCC/Ag/APC	<20	<20	<20	<20	502	<20		
		Ag/APC	<20	<20	<20	<20	502	<20		
TGF-β	SSRem104-3	anti-CD3	Dex	0	10⁻⁴	10⁻⁵	10⁻⁶	10⁻⁷	10⁻⁸	
		TCC/Ag/APC	780	3,800 (387)	2,700 (246)	900 (15)	820 (5)	820 (5)		
		Ag/APC	810	5,200 (542)	2,000 (147)	810 (0)	820 (1)	980 (21)		
	BPRem142-4	anti-CD3	0	70	0	0	0	40		
		TCC/Ag/APC	780	3,100 (297)	1,100 (41)	800 (3)	810 (4)	640 (-18)		
		Ag/APC	700	3,100 (343)	1,500 (114)	760 (9)	820 (17)	800 (14)		

^a Cytokine secretion by APCs in the absence of PLP-TCCs was minimal or undetectable, and remained unchanged in the presence of hormone.

^b Hormone doses are in bold type. For progesterone (Prog), concentrations are expressed as ng/ml, for estriol (E3) as pg/ml, and for dexamethasone (Dex) as M. Cytokine concentrations are expressed as pg/ml, except for TNF- $\alpha\beta$, which is expressed as U/ml.

^c Numbers in parentheses represent percent change in the presence of the indicated dose of hormone relative to cytokine values secreted in the absence of hormone.

Again, this effect was dose dependent, with maximal inhibition (70–85%) occurring at the highest concentration tested (10⁻⁴ mol/L). Eighty percent, or four of five of the TCCs tested were Dex responsive (Table II). Three of these clones showed almost identical dose-response patterns, while one (PK104-6) showed less overall sensitivity to Dex inhibition. Cell viability was \geq 93% after exposure to Dex concentrations from 10⁻⁴ to 10⁻⁸ mol/L, indicating that inhibition of IFN- γ and TNF- $\alpha\beta$ was not due to cell death.

In contrast to the inhibition of IFN- γ and TNF- $\alpha\beta$ release, Dex enhanced the secretion of TGF- β by PLP-TCCs in response to Ag/APC (Fig. 3, *e* and *f*; Table III). This enhanced production of TGF- β was observed only at high concentrations (10⁻⁴ to 10⁻⁵ mol/L), and occurred in all tested clones (8/8; Table II). Depending

upon the individual clone and experiment, maximal enhancement varied from approximately 250 to 650% at the highest concentration tested. Dex had no effect on the secretion of IL-4 and IL-10 (data not shown).

Steroid-responsive TCCs from MS patients and normal control subjects exhibited a similar degree of steroid sensitivity regardless of Th subset or disease status. In addition, the dose-response curves for E1, E3, Prog, and Dex effects on IL-4, IL-10, IFN- γ , TNF- $\alpha\beta$, and TGF- β did not change when PLP-TCCs were stimulated by anti-CD3 mAb in the absence of APCs (Table III). However, at equivalent hormone concentrations, there was a tendency for slightly higher concentrations of cytokines to be produced upon anti-CD3 mAb stimulation than those observed for cognate peptide Ag/APCs. These data suggest that it is unlikely that the APCs

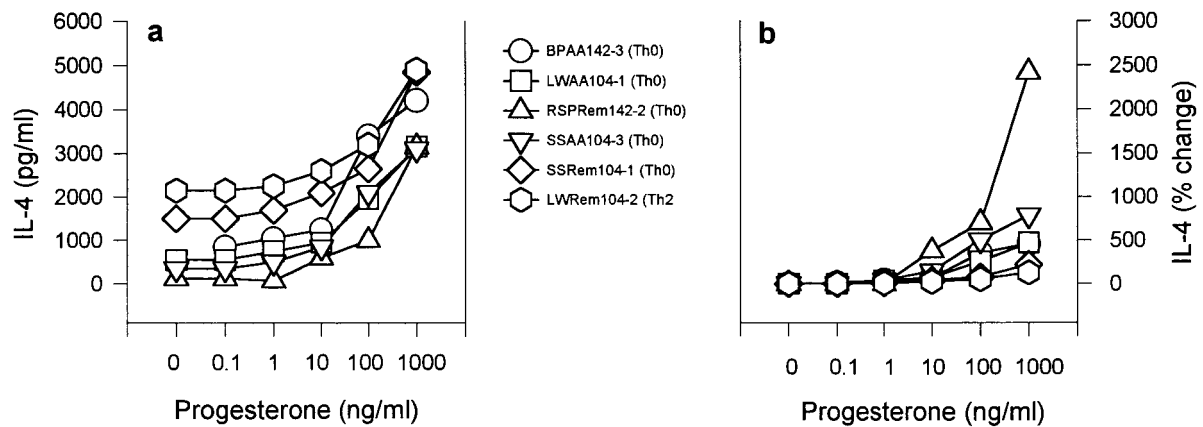


FIGURE 2. Prog enhances IL-4 secretion from PLP-TCCs in dose-dependent fashion. Supernatants were collected at 36 h after stimulation by Ag/APC in the presence and absence of Prog. IL-4 was measured by capture ELISA, as described in *Materials and Methods*. The data shown are representative of 12 PLP-TCCs that were tested at least twice following stimulation by Ag/APC or anti-CD3 mAb. All 12 clones exhibited a similar pattern of dose-dependent enhancement of Ag/APC-stimulated IL-4 secretion in the presence of Prog; six of these clones are included in *a* and *b*. The enhancement was particularly robust at 1000 ng/ml, but clearly evident at lower doses. The data in *a* are expressed as percentage of change in *b*.

contribute to or are responsible for the steroid influences on cytokine secretion in these studies. Moreover, Table III demonstrates that autologous irradiated adherent PBMC, which were used as APCs, secreted very low or undetectable levels of all of the cytokines tested after peptide stimulation in the absence of cloned T cells, and were not altered significantly by the addition of any hormone. Finally, the addition of ethanol at concentrations similar to those used for the dilution of steroid hormones did not affect cytokine secretion, which was also not changed when phenol red-free medium was used. These findings clearly indicate that the effects on cytokine secretion observed in these TCCs were due exclusively to the presence of steroid hormones, and not the result of technical artifacts.

Discussion

The data in this communication clearly indicate that estrogens and Prog can be added to the list of steroids, including glucocorticoids, androgens (adrenal and testicular), and 1, 25 (OH)₂ vitamin D₃, capable of modulating cytokine synthesis in the immune system (25–28). Since the final outcome of an immune response, whether or not it is beneficial to the host, is highly dependent upon the pattern of cytokines secreted upon contact with Ag, these findings imply that steroid hormones are capable of influencing the character of that immune response. Although the current experiments were not designed to determine whether E1, E3, Prog, or Dex is capable of inducing the secretion of a new pattern of cytokines or influencing the development of Th subsets, they were designed to identify their ability to up- or down-regulate cytokine secretion in cloned, Ag-specific T cells exhibiting well-established, stable cytokine secretion patterns. Thus, the data primarily have implications for steroid modulation of ongoing immune responses, and suggest that estrogens, Prog, and glucocorticoids individually and collectively support Th2-like activities at the high concentrations that are associated with pregnancy. This type of activity may be responsible for the changes in immune responsiveness that occur during gestation (22–24), and may also provide an explanation for the tendency for MS, rheumatoid arthritis, and autoimmune thyroid disease to remit during pregnancy (23, 37–39).

It is important to note that Piccinni et al. (40) found that high concentrations of Prog facilitate development of Th0- over that of Th1-like Ag-specific cells in vitro, in addition to enhancing secre-

tion of IL-4 and IL-5 and inducing expression of IL-4 mRNA in established Th1 clones. Although it is not known whether estrogens and corticosteroids act in a similar manner, these findings indicate potential for steroids to modulate Th1- or Th2-like development during earlier stages of responsiveness to Ag. Since IL-4 is critical to the development of Th2 cells (1–4), Prog appears to be a good candidate for their promotion. Moreover, responsiveness to Prog appears to be a relatively stable intrinsic property of T cells, since all of the clones tested in the current study showed enhancement of IL-4 in the presence of Prog.

It is quite evident that steroid concentration plays a crucial role in the regulation of cytokine secretion and, subsequently, in the functional significance of that regulation. For example, estrogens and Prog are most likely to exert a Th2-like influence only at concentrations associated with pregnancy (up to 35,000 pg/ml). Thus, at these doses and higher, IL-4 and IL-10 secretion were enhanced, and TNF- α inhibited. It is unlikely that glucocorticoids would affect immune function in healthy, nonpregnant individuals, since Dex inhibition of IFN- γ and TNF- α occurred at pharmacologic doses of 10⁻⁷ to 10⁻⁴ mol/L (equivalent to 1–1000 μ g/ml of cortisol, the natural human glucocorticoid). Enhancement of TGF- β was even more restricted to the highest tested doses (10⁻⁵ to 10⁻⁴ mol/L). Pregnancy-associated concentrations of cortisol range from 0.2 to 0.7 μ g/ml (41). Since steroid hormones accumulate at higher concentrations in target or source tissues than in the peripheral circulation, a localized Th2-like environment with potential for an impact on immunity is more likely to occur during pregnancy. However, lower doses of an individual steroid may be sufficient for a collective, or synergistic action of more than one steroid on cytokine secretion. This latter possibility can be easily tested using the PLP-TCCs, measuring cytokine secretion in the presence of combinations of lower doses of estrogens, Prog, and/or Dex. Finally, lower doses of estrogens alone (<5000 pg/ml) may actually promote Th1-like activity via enhancement of IFN- γ and TNF- α , in the absence of an opposing influence from IL-10, which requires higher concentrations for enhancement.

The fact that estrogens and Prog are capable of regulating cytokine secretion in T cells offers a possible explanation for the sexual dimorphism that has been observed in general immune function (42, 43). Thus, females tend to exhibit more effective immune responsiveness than males and are significantly more

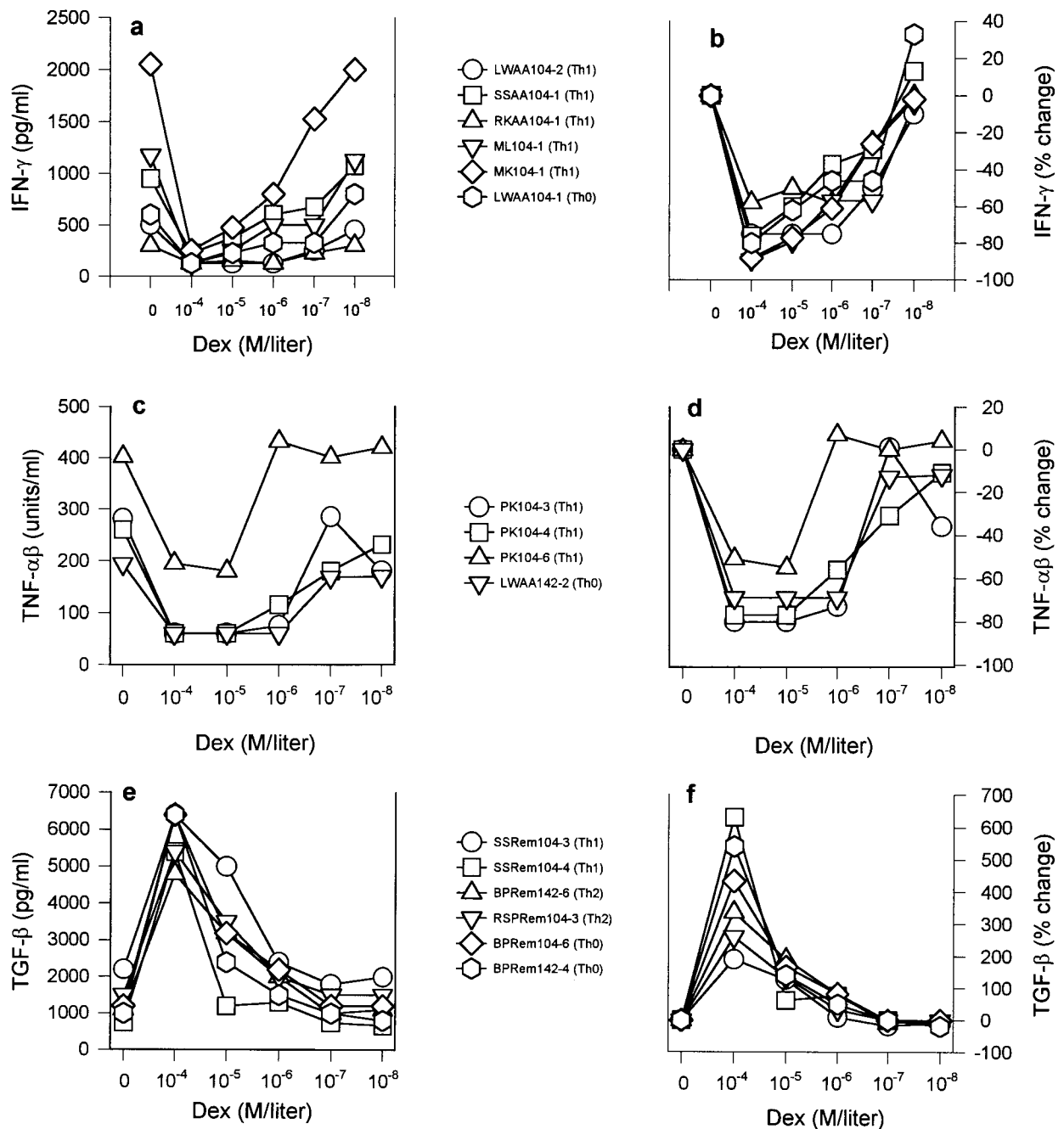


FIGURE 3. The effect of Dex on IFN- γ , TNF- α , and TGF- β secretion by PLP-TCCs. PLP-TCCs were stimulated by Ag/APC in the presence of varying concentrations of Dex, and supernatants were assayed at 72 h for IFN- γ (a and b), TNF- α (c and d), and TGF- β (e and f). The data in a, c, and e are expressed as percentage of change in, respectively, b, d, and f. IFN- γ secretion was inhibited in all tested clones ($n = 10$) in dose-dependent fashion, with most showing remarkably similar dose-response curves. Inhibition varied from 55 to 85% at 10^{-4} M/L and was minimal or did not occur at 10^{-7} and 10^{-8} M/L in most clones (b). Four of five PLP-TCCs also showed inhibition of TNF- α at 10^{-6} to 10^{-4} M/L. Percentage of inhibition was as high as -80% (d). All clones tested for an effect of Dex on TGF- β ($n = 8$) showed robust enhancement at 10^{-4} M/L, decreasing to minimum effects at 10^{-6} M/L and lower. All experiments testing the effect of Dex on cytokine secretion were repeated at least twice in PLP-TCCs stimulated by Ag/APC or anti-CD3.

prone to autoimmune diseases (42–44). An example of this type of sexual dimorphism is found in female susceptibility and male resistance to EAE in SJL mice (45). EAE susceptibility in females is accompanied by a bias for production of IFN- γ in neuroantigen-stimulated T cells, while cytokine secretion in T cells from resistant males is dominated by IL-4 and IL-10 (46–48). This is consistent with the ability of estrogens to enhance IFN- γ secretion at lower concentrations than required to enhance IL-10 secretion. Resistance to EAE can be induced in SJL females by s.c. implantation

of dihydrotestosterone-containing pellets (48). Since IL-10 production was enhanced in neuroantigen-specific T cells from treated females, these data suggest that male resistance is due to promotion of IL-10 secretion by testosterone.

The data in this communication also indicate that selective regulation of cytokines by steroid hormones occurs in cloned, Ag-specific human CD4⁺ T cells, apparently independent of T cell subset identity and regardless of the disease status or sex of the individual donor. Thus, the use of TCCs with Th0, Th1, or Th2

subset identity allowed us to determine that steroid-specific regulatory mechanisms appear not to be T cell subset specific, and most likely operate at the level of individual cytokine gene expression. If the steroid effects were TCC or subset specific, one would expect that all cytokines secreted by a single PLP-TCC would show sensitivity to the tested steroids. This did not occur. For example, IL-10, but not IL-4 secretion by clone BPAA142-3 was affected only by E1 and E3, while only IL-4 was sensitive to the presence of Prog. Similarly, if a clone secreted both IL-10 and IFN- γ , the secretion of both was modified by E1, E2 (32), and E3, but not Prog, regardless of the ability to secrete IL-4. Many PLP-TCCs were tested for cytokine sensitivity to more than one hormone (Table I), with the result that IL-10, IFN- γ , and TNF- $\alpha\beta$, and not IL-4 and TGF- β were sensitive to the estrogens, IL-4, and no other cytokine was sensitive to Prog, and IFN- γ , TNF- $\alpha\beta$, and TGF- β , but not IL-4 or IL-10, were affected by Dex (summarized in Table II). Thus, it is likely that individual cytokines, rather than specific T cell subsets, were the targets of the steroids tested in this study. However, definitive proof of this possibility requires a more mechanistic approach than used in this study. If proven to be correct, these data have implications for the mechanisms by which cytokine gene transcription is differentially or coordinately controlled in activated T cells, which are currently not well understood. One possibility is that steroid receptors, which classically act as ligand-activated nuclear transcription factors, bind to hormone response elements in the promoter regions of cytokine genes (49, 50). This mechanism is likely to be responsible for estrogen enhancement of IFN- γ , since it contains a functional ERE in its promoter (31). Examination of the 5' flanking region of the IL-10 promoter also reveals possible ERE sequences (51), although it is not known whether they are functional. Estrogen receptor-mediated signals may also converge upon the transcriptionally active complex of proteins, including NF-ATp/c (nuclear factor of activated T cells), members of the AP-1 (c-Fos, c-Jun) and NF- κ B (c-rel, p50, p65) families that are believed to be involved in cytokine gene expression in activated T cells (52–54). The inhibitory influence of Dex on IFN- γ and TNF- $\alpha\beta$ secretion may occur via inhibitory interactions between glucocorticoid receptors and AP-1 complexes or NF- κ B (50, 55, 56) or by the induction of I- κ B, an inhibitor of NF- κ B (50, 57). Since IL-4 secretion is a determinant and marker of the Th2 subset, it would be of particular interest to identify the mechanisms by which Prog induces its secretion.

There were several clones that consistently showed lack of responsiveness to E2 (32), E1, or E3, two of which were included in the current study (RP104-1 and JC104-3). Lack of estrogen sensitivity in these clones could not be explained by general resistance to steroids, since both showed enhanced IL-4 secretion in response to Prog. Estrogen insensitivity may reflect defective or unstable estrogen receptor expression or function, or perhaps phenotypic variability among CD4⁺ TCCs. It seems unlikely that cell cycle plays a primary role in determining the presence or absence of estrogen sensitivity, since 1) the majority of Ag/APC- or anti-CD3-stimulated PLP-TCCs showed estrogen responsiveness, 2) estrogen-insensitive clones were nevertheless sensitive to Prog, and 3) all tested PLP-TCCs showed changes in IFN- γ , TNF- $\alpha\beta$, or TGF- β in the presence of Dex. Cell cycle dynamics may, however, influence the magnitude of steroid sensitivity exhibited by the clones, and may explain the variability in cytokine secretion observed between clones. Finally, it is unlikely that the effect of steroids on cytokine secretion in these experiments was due to an influence on APCs, since they occurred upon stimulation of the PLP-TCCs by anti-CD3 mAb in the absence of APCs. Moreover, the autologous, irradiated adherent PBMC used as a source of APCs in these experiments secreted insignificant quantities of cytokines in the absence of clones, and showed no steroid responsiveness. These data

do not, however, exclude a role for APCs, and in fact, steroids have been well documented to modulate cytokine secretion in nonirradiated cells capable of acting as APCs (58–60).

Overall, the data reported in this communication suggest that the study of steroid influences on cytokine secretion has potential to contribute to our understanding of the mechanisms by which cytokine secretion is differentially or coordinately regulated. In addition, the data indicate a role for steroid hormones in regulating the development and function of CD4⁺ T cell subsets. Finally, these data provide encouragement for the continued testing of steroid hormones, alone or in combination, for potential use in the treatment of autoimmune diseases (61).

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