

Superantigen-induced corticosteroid resistance of human T cells occurs through activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK-ERK) pathway

Ling-bo Li, PhD,^a Elena Goleva, PhD,^a Clifton F. Hall, MS,^a Liang-Shiou Ou, MD,^c and Donald Y. M. Leung, MD, PhD^{a,b} Denver, Colo, and Taoyuan, Taiwan

Background: Microbial superantigens induce human T-cell resistance to corticosteroids.

Objective: Understanding the molecular pathways resulting in corticosteroid-resistant T cells is important because this condition can complicate the treatment of inflammation.

Methods: The response of human PBMCs to steroids was assessed by using proliferation assays after stimulation with superantigens or anti-CD3 in the presence of various kinase inhibitors. Glucocorticoid receptor α (GCR α) localization was defined on the basis of intracellular staining. Protein phosphorylation was measured by means of Western blotting.

Results: In the current study we found that PBMCs stimulated with superantigen, but not anti-CD3, induced corticosteroid-resistant T cells. However, the purified T cells stimulated either with staphylococcal enterotoxin B (SEB) or anti-CD3 are susceptible to corticosteroid inhibition. These results imply that signals on antigen-presenting cells might act in concert with the T-cell receptor to cause steroid resistance. Blockade of CD40–CD40 ligand interaction had no effect on superantigen-induced corticosteroid resistance. However, CD28 costimulation with T-cell receptor activation induced corticosteroid resistance of human T cells in a dose-dependent manner. Superantigen stimulation, compared with anti-CD3 stimulation, was found to induce a more rapid and sustained phosphorylation of mitogen-activated extracellular signal-regulated kinase (ERK).

Treatment with PD98059 and UO126 (specific mitogen-activated protein kinase kinase [MEK]/ERK inhibitors), but not a p38 inhibitor or a c-Jun N-terminal kinase inhibitor,

restored the response to steroids, as indicated by proliferation assays. Furthermore, purified ERK1 and ERK2 were able to phosphorylate recombinant human GCR α directly in an *in vitro* kinase assay. Of note, superantigen-induced corticosteroid resistance was associated with abrogation of GCR α nuclear translocation. This effect could be reversed by treatment with MEK/ERK pathway inhibitors.

Conclusions: These data are compatible with the hypothesis that superantigen-induced corticosteroid resistance involves the Raf-MEK-ERK1/ERK2 pathway of T-cell receptor signaling, which leads to GCR α phosphorylation and inhibition of dexamethasone-induced GCR α nuclear translocation. (J Allergy Clin Immunol 2004;114:1059–69.)

Key words: Superantigens, T cells, steroid resistance, MEK/ERK pathway, corticosteroids, glucocorticoid receptor

Microbial infection frequently triggers the onset and aggravates the course of chronic inflammatory diseases. An important mechanism by which microbes contribute to disease pathogenesis is through the secretion of superantigens. These molecules bind directly, without antigen processing, to MHC class II molecules on antigen-presenting cells (APCs) and trigger the potent activation of T cells through selected T-cell receptor β (TCR- β) variable region elements.¹ TCR repertoire analysis of PBMCs or cells from sites of local tissue inflammation have demonstrated involvement of superantigens in the pathogenesis of a number of diseases, including Kawasaki disease, rheumatoid arthritis, severe asthma, atopic dermatitis, and guttate psoriasis.^{2–6}

Corticosteroids are the mainstay of anti-inflammatory therapy in many chronic diseases. However, a subset of patients who fail to respond to corticosteroids have been referred to as corticosteroid resistant (CR).⁷ For example, a recent study in the Journal revealed that an analysis of T cells from the bronchoalveolar samples of patients with severe asthma who did not respond well to corticosteroids was consistent with an expansion of their TCR- β chain consistent with superantigen stimulation.⁴ Interestingly, in Kawasaki disease intravenous immunoglobulin, rather than corticosteroids, is first-line therapy.⁸ Furthermore, atopic dermatitis colonized with *Staphylococcus aureus* is

From ^athe Department of Pediatrics, National Jewish Medical and Research Center, Denver; ^bthe Department of Pediatrics, University of Colorado Health Sciences Center, Denver; and ^cthe Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, Chang Gung Children's Hospital, Taoyuan.

Dr Leung's work was supported in part by National Institutes of Health grants HL36577, AR41256, and HL37260; the Ann and Louis Rudolph Kawasaki Disease Research Fund; and the Edelstein Family Chair in Pediatric Allergy and Immunology, the University of Colorado Cancer Center.

Received for publication May 8, 2004; revised August 4, 2004; accepted for publication August 5, 2004.

Available online October 7, 2004.

Reprint requests: Donald Y. M. Leung, MD, PhD, National Jewish Medical and Research Center, 1400 Jackson St, Room K926i, Denver, CO 80206. E-mail: leungd@njc.org.

0091-6749/\$30.00

© 2004 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2004.08.005

Abbreviations used

APC:	Antigen-presenting cell
CD40L:	CD40 ligand
CR:	Corticosteroid resistant
Cy3:	Indocarbocyanine
DAPI:	4'-6-diamidino-2-phenylindole, dihydrochloride
ERK:	Extracellular signal-regulated kinase
GCR:	Glucocorticoid receptor
IC ₅₀ :	Inhibitory concentration of 50%
JNK:	c-Jun N-terminal kinase
MAPK:	Mitogen-activated protein kinase
MEK:	Mitogen-activated protein kinase kinase
MFI:	Mean fluorescence intensity
PI3K:	Phosphatidylinositol 3-kinase
PKC:	Protein tyrosine kinase C
SEB:	Staphylococcal enterotoxin B
TCR:	T-cell receptor
TSST-1:	Toxic shock syndrome toxin 1

more responsive to treatment with the combination of corticosteroids and antibiotics compared with corticosteroids alone.⁹ Although the role of superantigens in these latter 2 diseases is still debated, it is of interest that intravenous immunoglobulin has high titers of antibodies against staphylococcal superantigens, and antibiotics act to shut off superantigen production by *S aureus*.^{10,11} In the case of patients with severe asthma, rheumatoid arthritis, and psoriasis, failure to control inflammation with corticosteroid therapy can lead to disease progression. Therefore it is important to understand mechanisms underlying CR conditions and develop new therapeutic targets for intervention in this process. A recent study demonstrated that superantigens can induce CR T cells *in vitro*; however, the molecular mechanism for this effect has not been elucidated.¹² In the current study we addressed the hypothesis that development of T-cell resistance to corticosteroids after superantigen activation occurs as the result of activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway.

METHODS**Reagents**

Staphylococcal enterotoxin B (SEB), toxic shock syndrome toxin 1 (TSST-1; Toxin Technology, Sarasota, Fla), anti-human CD3 (Orthoclone OKT 3 sterile solution; Ortho Biotech Products, L.P., Raritan, NJ), CD28 (BD PharMingen, San Diego, Calif), and dexamethasone (Sigma Chemicals Co, St Louis, Mo) were used for cell stimulation. The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (inhibitory concentration of 50% [IC₅₀] = 35 nM), the MEK inhibitors U0126 (IC₅₀ = 72 nM) and PD98059 (IC₅₀ = 2 μM), c-Jun N-terminal kinase (JNK) inhibitor (IC₅₀ = 40 nM), the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (IC₅₀ = 5 nM), the phosphokinase C (PKC) inhibitor RO-31-8220 (IC₅₀ = 10 nM), and the PKCθ inhibitor TER 14687 were obtained from Calbiochem (San Diego, Calif). Fluorochrome-labeled CD3 (clone SK7), CD14 (clone M5E2), and CD19 (clone HIB19) antibodies were purchased from BD PharMingen. Purified anti-CD40 ligand (anti-

CD40L) antibody and human CD154 (CD40L) μCD8 fusion protein (Anncell Corp, Bayport, Me) were used in blocking experiments.

Isolation of human PBMCs

PBMCs were isolated by means of Ficoll-Hypaque density gradient centrifugation from heparinized venous blood obtained from healthy donors, as previously described.¹² Cultures were subsequently stimulated with 100 ng/mL SEB, 100 ng/mL TSST-1, or 0.5 μg/mL soluble anti-CD3 and maintained for the indicated time.

Cell sorting

CD3⁺, CD14⁺, and CD19⁺ cell populations were sorted on the Mo-Flow cytometer (Cytomation, Fort Collins, Colo) on the basis of surface marker expression. The purity of isolated cells was 99%, as determined by means of flow cytometric analysis.

Intracellular staining for glucocorticoid receptor

Glucocorticoid receptor α (GCRα) nuclear translocation in response to dexamethasone was analyzed by means of fluorescence microscopy, as previously described.¹³ In brief, human PBMCs (1 × 10⁶ cells/mL) stimulated with superantigens or anti-CD3 for 48 hours were subsequently treated with 10⁻⁶ M dexamethasone for 1 hour or remained untreated. The GCR intracellular staining was performed as described earlier.¹³ The anti-GCR antibody (PA1-516; Affinity Bioreagents, Golden, Colo) used in this study recognizes only GCRα. Intensity of GCRα staining was assessed by using image analysis software (Slidebook; Intelligent Imaging Innovations, Denver, Colo) and expressed as mean fluorescence intensity (MFI). Fifty to 100 cells were analyzed per slide. In brief, a series of 1-μm z-frames were taken through groups of cells in both the 4'-6-diamidino-2-phenylindole, dihydrochloride (DAPI) (nuclear) and indocarbocyanine (Cy3) (GCRα) channels. Each stack of images then underwent the nearest neighbor's deconvolution. This allowed for exact localization of the nucleus within the cell and removed background fluorescence from above and below the z-frame that could affect the reliability of the results. A single computer-generated mask was then defined on the basis of the DAPI staining. The intensity of Cy3 staining (GCRα) was assessed by using the analysis software purely within the DAPI mask and hence the nucleus and the Cy3 mask, from which the DAPI mask was subtracted (cytoplasm). In this way increased MFI within DAPI masks (cell nuclei) corresponds to increases in GCRα within the nucleus and hence receptor nuclear translocation. The data can be as well presented as a nuclear/cytoplasm ratio of corresponding Cy3 masks for nuclear and cytoplasmic regions of cells that were analyzed.

For GCR nuclear translocation experiments in the presence of inhibitors and blocking antibodies, the cells were preincubated with these reagents for 2 hours at 37°C before superantigen stimulation and then treated as described above or used for cell proliferation assays. All inhibitors and blocking antibodies remained in cell culture after preincubation.

Cell proliferation assays

Cell proliferation assays were performed in 96-well round-bottom plates in complete medium: RPMI-1640 (Cellgro; Mediatech, Inc, Herndon, Va) supplemented with 10% FBS (Gemini Bio Products, Calabasas, Calif), 1% penicillin-streptomycin, and 20 mM HEPES (Mediatech, Inc). Human PBMCs (1 × 10⁵/well) and purified T cells (7-9 × 10⁴/well) in a total volume of 200 μL were stimulated with 100 ng/mL SEB or 0.5 μg/mL anti-CD3 with or without 0.001 to 1 μg/mL soluble anti-CD28 antibodies, as previously described,¹² in the absence or presence of 10⁻⁹ to 10⁻⁶ M dexamethasone. For dose-response experiments, 4 different concentrations of anti-CD3

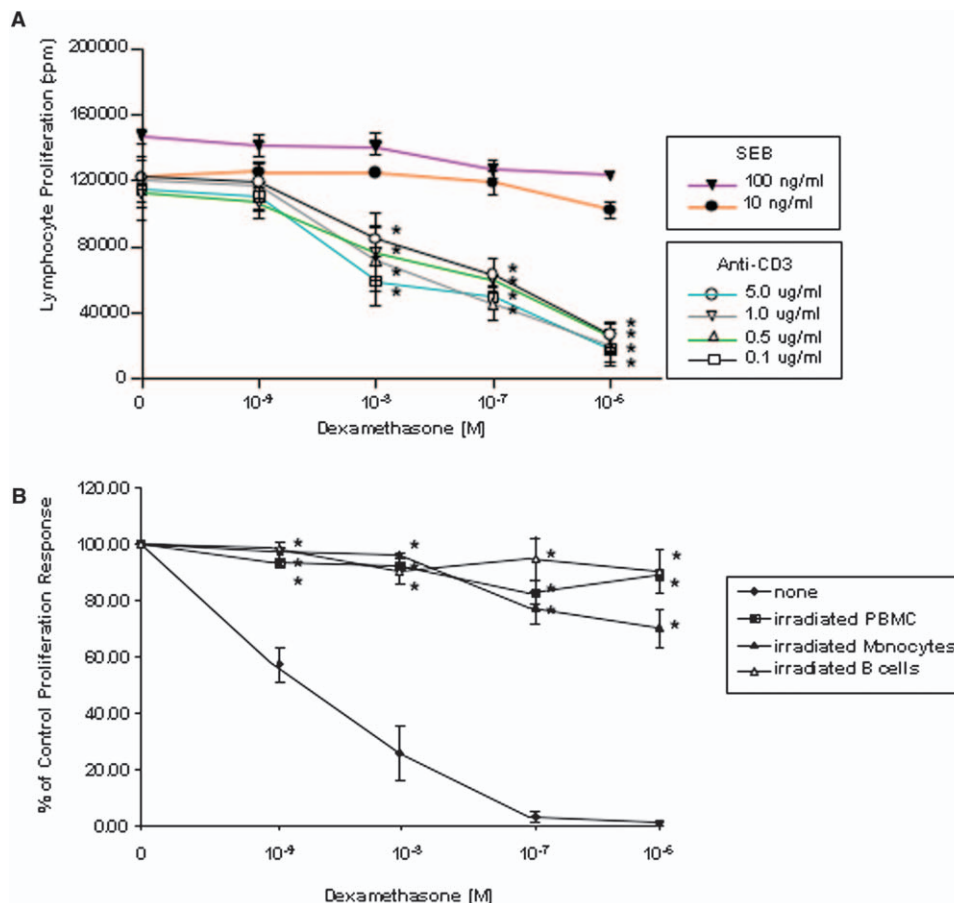


FIG 1. Requirements for delivery of CR response through the TCR. **A**, Proliferative response of SEB (superantigen)- or anti-CD3-stimulated PBMCs to dexamethasone ($n = 4$). * $P < .001$ compared with cells cultured in the absence of dexamethasone. Results are expressed as mean $\pm$ SEM counts per minute of triplicate cultures. **B**, Response of purified T cells to SEB in the presence of steroids and requirement of APCs for the development of steroid resistance. * $P < .01$ compared with purified T cells stimulated with superantigen without APCs. Proliferative response data under various conditions are expressed as a percentage of superantigen-induced tritiated thymidine incorporation in the absence of dexamethasone.

(0.1 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, and 5 $\mu\text{g/mL}$) and SEB (10 ng/mL , 100 ng/mL , 500 ng/mL , and 1 $\mu\text{g/mL}$) were used. The cultures were incubated for 72 hours at 37°C in a humidified 5% CO_2 incubator. The cell proliferation was assessed on the basis of tritiated thymidine incorporation, as previously described.¹³

It has been shown that immobilized superantigens can be used to stimulate T-cell proliferation in the absence of APCs.¹⁴ Therefore in some experiments the purified CD3^+ T cells were cultured in plates precoated with SEB (5 $\mu\text{g/mL}$ SEB in 0.1 M sodium carbonate, pH 9.6, buffer overnight at 4°C) or with 100 ng/mL soluble SEB in combination with 0.001 to 1 $\mu\text{g/mL}$ anti-CD28. When soluble SEB was used for T-cell stimulation, the cell cultures were supplemented with purified monocytes, B cells, or PBMCs irradiated at 3000 rad.

Western blot analysis

Human PBMCs were incubated with 100 ng/mL SEB and 0.5 $\mu\text{g/mL}$ anti-CD3 with or without 0.5 $\mu\text{g/mL}$ anti-CD28 for different time points and lysed in ice-cold TENT lysis buffer with protease inhibitors cocktail (Sigma). For experiments with inhibitor treatment, cells were preincubated with inhibitors for 1 hour or with dimethyl sulfoxide as a solvent control and then stimulated with mitogens still in the presence of inhibitors. Thirty micrograms of total protein per

condition were resolved on 12% SDS-PAGE gel and transferred to nitrocellulose membrane (Bio-Rad, Hercules, Calif). The membranes were blocked in Tris-buffered saline/5% dry milk/0.05% Tween 20 for 2 hours and incubated with anti-phospho-ERK or anti-ERK antibodies (1:5000; Santa Cruz Biotechnologies, Santa Cruz, Calif) at 4°C overnight. Subsequently, membranes were washed in Tris-buffered saline/0.05% Tween 20 and incubated for 1 hour at room temperature with anti-mouse or anti-rabbit horseradish peroxidase (HRP)-labeled IgG (1:500; Amersham Pharmacia Biotech, Piscataway, NJ), washed, incubated with chemiluminescent reagent (Perkin Elmer Life Sciences, Boston, Mass), and processed for autoradiography.

GCR phosphorylation

Recombinant human $\text{GCR}\alpha$ (1.25–20 μg , Affinity Bioreagents) was mixed with 10 ng of recombinant ERK1 or 25 ng of ERK2 kinases (Upstate, Charlottesville, Va) in assay dilution buffer I (Upstate). Twenty micrograms of myelin basic protein (Upstate) was used as a control substrate for the phosphorylation. Phosphorous 32 (^{32}P)-ATP cocktail (10 μCi) was added: 1 $\text{mCi}/100 \mu\text{L}$ (3000 Ci/mmol) [^{32}P]-ATP stock (Amersham Pharmacia Biotech) was diluted 1:10 with magnesium-ATP cocktail (75 mmol/L $\text{MgCl}_2/500 \mu\text{M}$

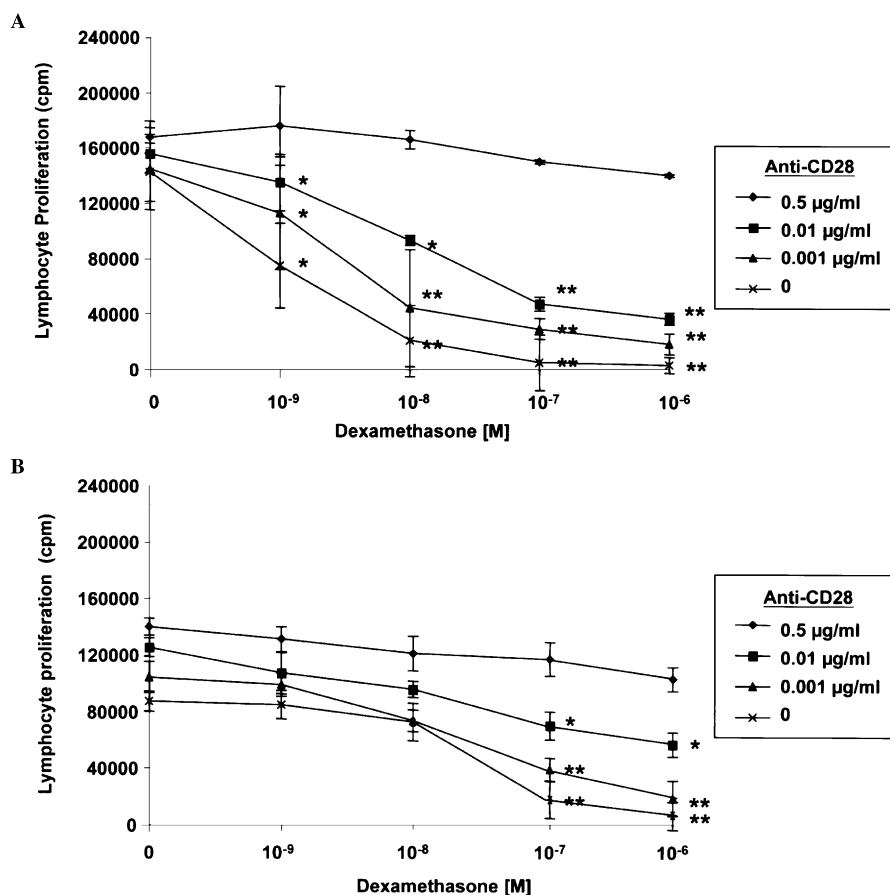


FIG 2. Proliferative response of T cells stimulated in the presence of increasing anti-CD28 costimulation after anti-CD3 stimulation of PBMCs at different concentrations of dexamethasone (**A**; $n = 5$, $*P < .01$ and $**P < .001$ compared with stimulation without dexamethasone) and after SEB stimulation of purified human T cells in an APC-free environment in the presence of different concentrations of dexamethasone (**B**; $n = 5$, $*P < .05$ and $**P < .001$ compared with stimulation without dexamethasone).

ATP in assay dilution buffer I) before the assay. The samples were incubated for 10 minutes at 30°C in a shaking incubator. Twenty-five microliters of samples was spotted onto P81 filter paper, washed 3 times with 0.75% phosphoric acid, and then washed once with acetone and counted in a liquid scintillation counter. The leftover samples were boiled in SDS sample buffer, resolved on 4% to 15% Tris-HCl gel (Bio-Rad) under denaturing conditions and then dried on thick filter paper in a vacuum drier for 1 hour at 65°C. The dry gels were processed by phosphorimaging to detect the [³²P] incorporation.

Statistical analysis

Immunofluorescence and cell proliferation data were analyzed by using the Student *t* test. A *P* value of less than .05 was considered significant. Data were expressed as means $\pm$ SEM.

RESULTS

Cellular requirements for superantigen-induced CR T cells

Because superantigens are known to activate T cells through the TCR, our initial experiments compared the effects of dexamethasone on superantigen (SEB or

TSST-1) versus anti-CD3-induced proliferation of human T cells. As shown in Fig 1, *A*, dexamethasone significantly inhibited the T-cell proliferation induced by anti-CD3. In contrast, SEB-induced (Fig 1, *A*) or TSST-induced (data not shown) T-cell proliferation was not affected by dexamethasone. This difference in steroid responsiveness between SEB versus anti-CD3 appeared to be due to a qualitative difference in signaling because SEB stimulation, compared with anti-CD3 stimulation, was steroid resistant over a wide range of concentrations, despite the use of lower concentrations of SEB than anti-CD3. This was not dependent on the magnitude of baseline proliferation (in the absence of dexamethasone) because SEB proliferation at 10 ng/mL was similar to the level of proliferation induced by 0.1 to 5 µg/mL anti-CD3. However, T cells at all concentrations of anti-CD3 tested were markedly more steroid sensitive than even T cells stimulated with low concentrations of SEB (ie, 10 ng/mL; $P < .001$).

Because TCR activation occurs through CD3, this set of experiments suggested that costimulatory signals by APCs might be needed for induction of CR T cells by

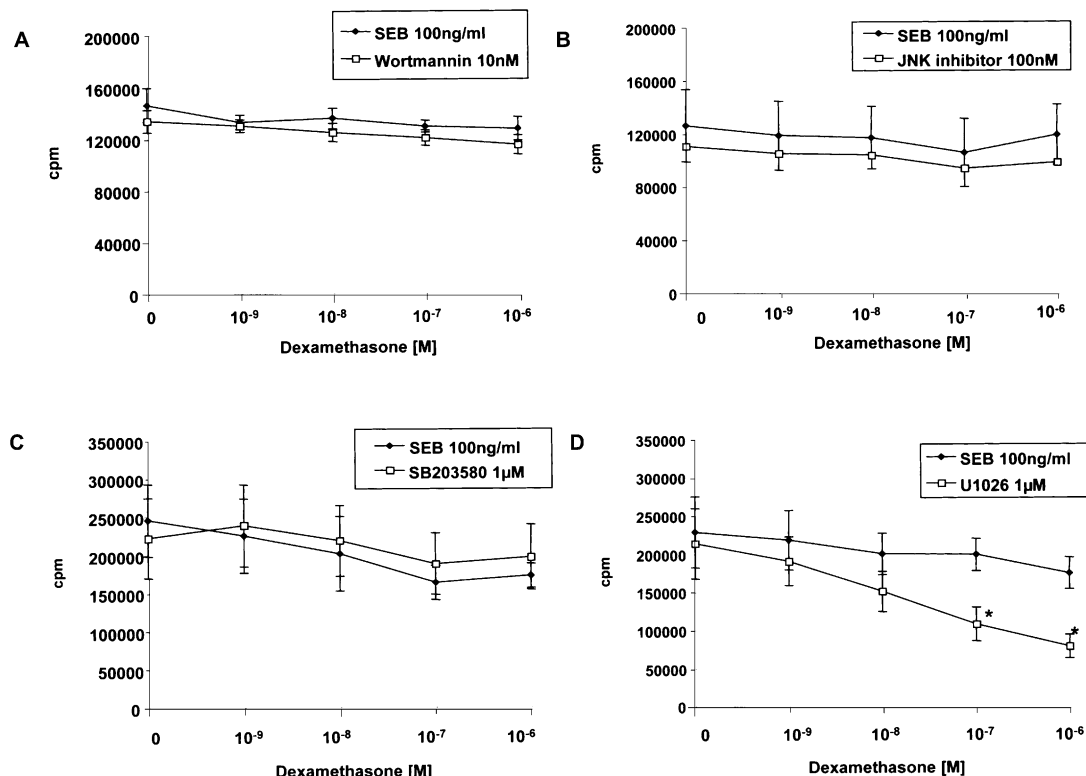


FIG 3. The effects of different protein kinase inhibitors on superantigen-induced corticosteroid resistance. Wortmannin (A), JNK inhibitor (B), or SB203580 (C) did not change superantigen-induced PBMC reactivity to dexamethasone. D, U1026 abrogates the CR effect induced in PBMCs by superantigens (* $P < .05$).

superantigens. Purified T cells were cultured with SEB alone or in the presence of APCs (irradiated PBMCs, B cells, or monocytes) to confirm the requirement of APCs. The effect of dexamethasone on T-cell proliferation induced by superantigen was assessed on the basis of tritiated thymidine incorporation. T cells can proliferate in response to plastic-immobilized superantigen in the absence of APCs¹⁴ because plastic-anchored SEB facilitates TCR cross-linking. As shown in Fig 1, B, when purified T cells (in the absence of APCs) were cultured in the plates precoated with SEB, dexamethasone significantly inhibited T-cell proliferation: 10^{-6} M dexamethasone caused up to 99% inhibition of the T-cell proliferation compared with SEB alone ($P < .0001$). In contrast, SEB-induced T-cell proliferation was significantly less inhibited by dexamethasone in the presence of APCs (irradiated PBMCs, monocytes, or B cells): the percentage of inhibition of cell proliferation was $11.39\% \pm 6.47\%$ ($P < .01$), $29.91\% \pm 7.63\%$ ($P < .01$), and $10.11\% \pm 0.94\%$ ($P < .01$), respectively (compared with that without APCs).

CD28, but not CD40, is involved in superantigen-induced corticosteroid resistance

Interactions between CD40 on APCs and its ligand on T cells has been implicated in T cell-mediated immune

responses.¹⁵ We therefore examined the role of the CD40-CD40L costimulatory pathway in this process. In these experiments PBMCs were preincubated with 2 to 10 $\mu\text{g/mL}$ CD40L blocking antibody or with CD40L fusion protein for 2 hours and then stimulated with 100 ng/mL SEB in the presence of different concentrations of dexamethasone still in the presence of blocking reagents. The effect of blocking antibody on T-cell reactivity to dexamethasone was compared with that of SEB alone. Disruption of CD40-CD40L interaction had no effect on superantigen-induced cell proliferation in the presence of dexamethasone (T-cell proliferation induced by SEB was not inhibited by the presence of the blocking antibody, even with 10^{-6} M dexamethasone: $142,316 \pm 6252$ cpm vs $148,448 \pm 7475$ cpm, respectively), indicating that other costimulatory signals must be involved in this process. The CD40L fusion construct also had no effect on superantigen-induced CR T cells (data not shown).

CD28 is known to provide a costimulatory signal that cooperates with the TCR/CD3 complex to induce T-cell activation.¹⁶ T cells were stimulated through the TCR by means of anti-CD3 cross-linking with or without different concentrations of anti-CD28 antibody to investigate the role of CD28 in the induction of CR T cells. Dexamethasone suppressed the ability of PBMCs to proliferate in response to anti-CD3 (Fig 2, A). Dexamethasone (10^{-6} M) caused a 90% inhibition of anti-CD3-induced PBMC

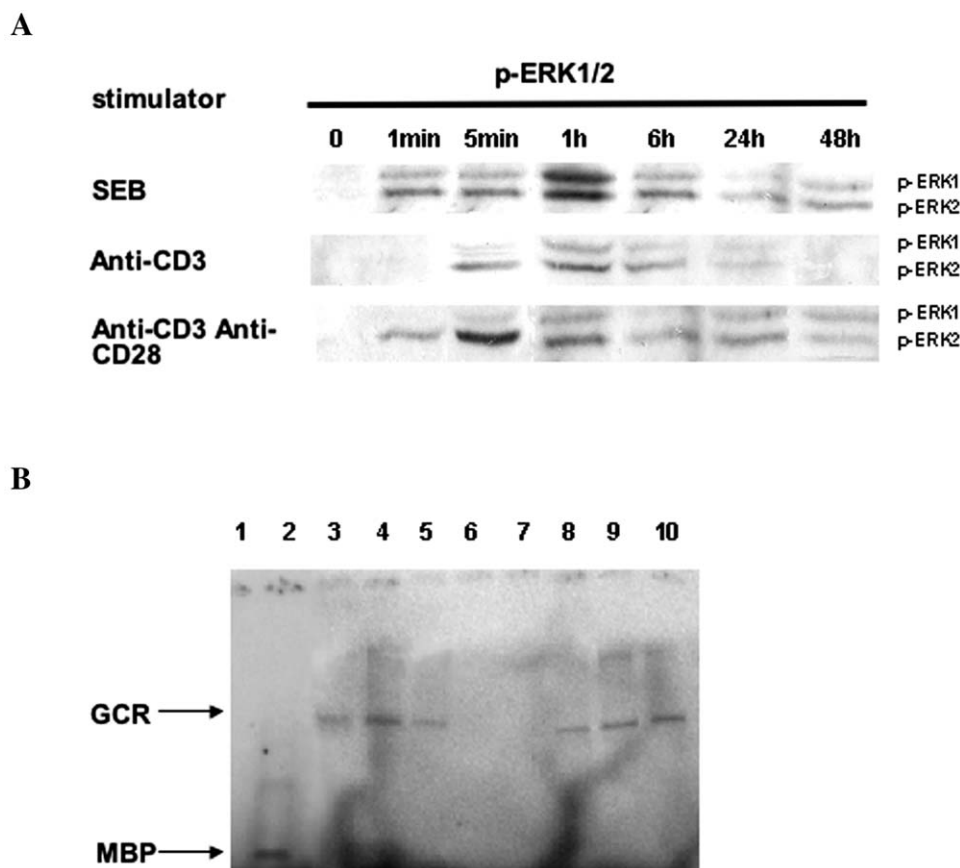


FIG 4. SEB, compared with anti-CD3, induces more rapid and sustained ERK activation. **A**, The kinetics of ERK phosphorylation after SEB versus anti-CD3 stimulation, as assessed by means of Western blotting. A representative experiment is shown of 3 independent experiments performed. **B**, *In vitro* phosphorylation of the recombinant human GCR (1 μ g, 5 μ g, and 20 μ g, respectively) by ERK1 (lanes 3-5) and ERK2 kinases (lanes 8-10). Lane 1, Blank; lane 2, positive control (myelin basic protein [MBP]); lanes 6 and 7, autophosphorylation controls for ERK1 and ERK2. MBP, Myelin basic protein.

proliferation. However, in the presence of increasing concentrations of soluble anti-CD28 antibody, T cells became more resistant to dexamethasone: at low concentrations of anti-CD28 (0.001–0.01 μ g/mL), the cells remained sensitive to dexamethasone, and higher concentrations of anti-CD28 (0.1–0.5 μ g/mL) restored T-cell proliferation, even in media with 10^{-6} M dexamethasone (Fig 2, A). These data suggested that CD28 costimulation shifted the response of human T cells to dexamethasone in a dose-dependent manner from a steroid-sensitive state to a CR state after stimulation through the TCR.

To further investigate the role of CD28 signaling in SEB-induced human T-cell steroid resistance, we examined the ability of an mAb to CD28 for its direct ability to act as a costimulatory signal for SEB in the absence of APCs. Purified T cells were cultured with anti-CD28 antibody (0.001–1.0 μ g/mL) for 72 hours in the presence of SEB. In the control group (SEB-coated plate) dexamethasone treatment suppressed the ability of T cells to proliferate in response to SEB (Fig 2, B). However, when T cells were stimulated with SEB in the presence of increasing concentrations of anti-CD28, T cells became

more CR. At 0.5 μ g/mL, anti-CD28 completely restored T-cell resistance to dexamethasone induced by SEB.

Involvement of the ERK pathway in superantigen-induced CR T cells

Previous experiments suggest that the CD28 costimulatory signal is important for the development of CR T cells induced by superantigen. To study the signaling pathways that link CD28 to superantigen induction of CR T cells, we first used a cell-permeable low-molecular-weight inhibitor specific for the PI3K wortmannin. Its effect was assessed by the ability of the inhibitor to restore inhibition of PBMC proliferation by dexamethasone after SEB stimulation. Surprisingly, PI3K inhibitor had no effect on SEB-induced steroid resistance (Fig 3, A). Because PKC cascade activation is considered to be the major downstream event after TCR engagement, we also tested the effects of selective PKC inhibitors: 100 nM RO-318220 (IC_{50} = 10 nM) and 0.5 μ M TER 14687. They did not subvert the response to corticosteroids as well (data not shown).

Previous studies have shown that CD28 plays a crucial role in superantigen-induced T-cell activation, and CD28

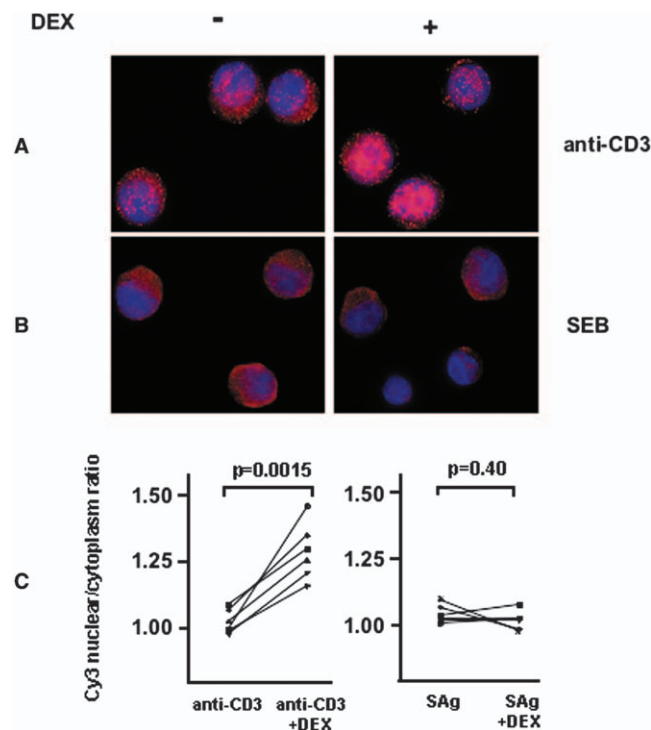


FIG 5. GCR nuclear translocation is inhibited after superantigen stimulation in the presence of dexamethasone (Original magnification 630 $\times$; blue, DAPI-nuclear staining; red, Cy3-GCR.) **A**, Anti-CD3–stimulated cells with (+) or without (–) dexamethasone treatment. **B**, SEB- or TSST-1–stimulated cells with (+) or without (–) dexamethasone treatment. The pictures are representative of 6 independent experiments in which 50 to 100 cells per slide were analyzed. **C**, Cy3 GCR nuclear/cytoplasm ratio in the cells stimulated with anti-CD3 and superantigens.

can augment signals from the TCR to the MAPK cascade.^{16,17} Therefore we sought to determine whether each of these signaling pathways had distinct effects on the molecular mechanisms that control steroid response.

PBMCs were preincubated with the specific p38 inhibitor SB203580, the JNK inhibitor, and the MEK/ERK inhibitor U0126 for 2 hours before SEB stimulation for 72 hours (all inhibitors remained in the cell culture). Several concentrations of the inhibitors were tested to determine the concentration that was not toxic to the cells and was not able to inhibit cell proliferation on its own. Data summarized in Fig 3, B and C, showed that 100 nM of JNK inhibitor (IC_{50} = 40 nM) and 1 μ M of p38 kinase inhibitor SB203580 (IC_{50} = 35 nM) had no effect on SEB-induced steroid resistance. However, the specific MEK/ERK inhibitor U0126 at 1 μ M (IC_{50} = 72 nM), which we verified by means of Western blotting with phospho-ERK antibodies to completely inhibit ERK phosphorylation (data not shown), partially restored the T-cell response to dexamethasone: 10^{-6} M dexamethasone caused up to $45.85\% \pm 13.12\%$ inhibition of the T-cell proliferation after SEB stimulation in the presence of U0126 inhibitor ($P < .05$ compared with SEB stimulation only in the presence of 10^{-6} M dexamethasone; Fig 3, D). This was further verified by another MEK/ERK inhibitor: 10 μ M PD98059 (IC_{50} = 2 μ M) caused up to $44.13\% \pm 10.58\%$

inhibition of SEB-induced T-cell proliferation in the presence of 10^{-6} M dexamethasone ($P < .05$ compared with SEB stimulation only in the presence of 10^{-6} M dexamethasone). In the presence of 10^{-6} M dexamethasone in the culture media, the counts were as follows: $55,406 \pm 5231$ cpm for SEB stimulation in the presence of 10 μ M PD98059 versus $143,176 \pm 35636$ cpm for SEB only ($P < .05$).

GCR α phosphorylation by ERK1/ERK2 kinases

Our experiments with MEK/ERK inhibitors strongly supported the involvement of ERK1/ERK2 in superantigen-induced steroid resistance. We further studied the relationship between superantigen stimulation and ERK phosphorylation at serial time points up to 48 hours. As shown in Fig 4, A, SEB induced ERK phosphorylation more rapidly (detectable within 1 minute) than the steroid-sensitive stimulator anti-CD3 (which required 5 minutes). After SEB stimulation, compared with anti-CD3 stimulation, ERK phosphorylation was also stronger and more persistent at the 48-hour time point. In contrast, phospho-ERK was not detectable after 48 hours of stimulation of PBMCs with anti-CD3. However, enhancement of costimulatory signaling by adding anti-CD28 to anti-CD3 quickly induced ERK phosphorylation (Fig 4, A). All groups expressed similar amounts of total ERK (see

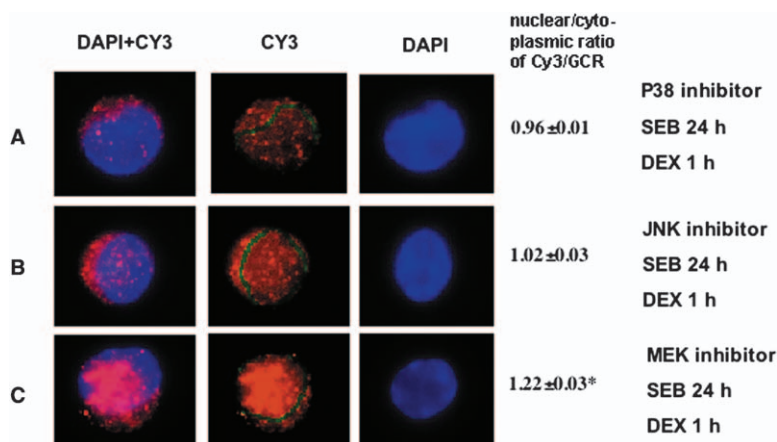


FIG 6. MEK inhibitor, but not p38 MAPK or JNK inhibitor, restores the GCR nuclear translocation of T cells in the presence of superantigen after dexamethasone treatment. Each panel is representative of 3 independent experiments in which 50 to 100 cells per slide were analyzed. **A**, p38 MAPK inhibitor (1 μ M). **B**, JNK inhibitor (100 nM). **C**, U0126 (1 μ M). * $P < .01$ compared with p38 MAPK- and JNK-treated cells that were stimulated with 10^{-6} M dexamethasone for 1 hour.

Fig E1 at the Journal's Online Repository at www.mosby.com/jaci.

It is known that GCR phosphorylation can be associated with a reduction in its activity in T-cell responses.¹⁸⁻²⁰ Indeed, when we treated purified recombinant human GCR α *in vitro* with purified ERK1 and ERK2 kinases, there was a significant increase in phosphorylation of the GCR α molecule (Fig 4, B). The degree of [^{32} P] incorporation was similar to [^{32} P] incorporation in the control substrate (myelin basic protein) after kinase phosphorylation (Fig 4, B, lane 2).

Superantigen stimulation prevents nuclear translocation of GCR in human T cells

It has been suggested that phosphorylation of the GCR influences its ability to translocate to the nuclear region in response to a receptor agonist.¹⁸⁻²⁰ Because we demonstrated in Fig 4, B, that ERK directly phosphorylates GCR, it was of interest to examine the effect of superantigen on GCR nuclear translocation. PBMCs from 6 healthy donors were stimulated with 100 ng/mL SEB or anti-CD3 for 48 hours. The cells were then treated with 10^{-6} M dexamethasone for 1 hour at 37°C or sham treated. In these experiments GCR α location was examined by means of indirect immunofluorescence with anti-GCR α rabbit polyclonal antiserum detected by means of Cy3-conjugated secondary antibody (red), and the cell nuclei were defined by staining with the blue fluorescent DNA dye DAPI. In the current study the response of SEB-stimulated T cells to steroids was evaluated by means of GCR α nuclear translocation. T cells were distinguished from monocytes by means of surface staining with anti-CD3 APC antibody.

In the absence of dexamethasone stimulation, GCR α was distributed uniformly in the cells (Fig 5, A and B). After dexamethasone treatment, GCR α translocated to the cell nucleus in all anti-CD3-stimulated cells (Fig 5, A), but

GCR failed to translocate into nuclei in SEB-stimulated cells (Fig 5, B). The nuclear/cytoplasm ratio of GCR α was 1.30 ± 0.09 in anti-CD3-stimulated cells after dexamethasone treatment ($P = .0015$ compared with the baseline nuclear/cytoplasm ratio of GCR α); in contrast, the nuclear/cytoplasm ratio of GCR α in SEB-stimulated cells was 1.02 ± 0.05 , which was not significantly different from GCR α distribution before dexamethasone treatment (1.04 ± 0.04 ; Fig 5, C). These data indicate that superantigen stimulation prevents nuclear translocation of GCR α induced by dexamethasone.

We then compared the effect of different MAPK inhibitors on GCR α nuclear translocation after SEB stimulation. In these experiments we treated the cells with the inhibitors in the same way and the same concentration as used for cell proliferation. In the SB203580- and JNK inhibitor-treated groups, dexamethasone had no effect on GCR α distribution (Fig 6, A and B). In contrast, dexamethasone induced efficient GCR α translocation in the U0126/SEB-treated group (Fig 6, C). Cy3 MFIs for the nuclear region of the cells before and after dexamethasone treatment in this group were as follows: 545.0 ± 215.3 versus 897.8 ± 287.12 ($P = .001$; $P < .01$ compared with p38 MAPK- and JNK-treated cells that were stimulated with 10^{-6} M dexamethasone for 1 hour).

DISCUSSION

The anti-inflammatory and immunosuppressive properties of corticosteroids are their major pharmacologic benefits and make them the most widely prescribed class of drugs in the world. The physiologic response and sensitivity to corticosteroids varies among individuals, tissues, and cell types.⁷ As such, corticosteroid resistance complicates the treatment of chronic inflammatory diseases. Although not proved, we hypothesize that superantigens contribute to the severity of various diseases by

inducing corticosteroid resistance.^{4,9,12,21,22} However, the molecular basis of glucocorticoid resistance is not well understood.⁷ In this article we demonstrate that activation of the MEK/ERK pathway is the key mediator and signaling pathway involved in the induction of superantigen-induced CR T cells.

Our study demonstrated that stimulation through the TCR/CD3 complex *per se* does not induce corticosteroid resistance. Costimulatory signals on APCs play a critical role in this process by augmenting the strength and duration of ERK activation. The interaction between CD28 on the T-cell surface and CD80/CD86 on the APCs, as well as the interaction of CD40 on the APCs and CD154 (CD40L) on the T-cell surface, are required for optimal activation of T cells by means of both conventional antigen and superantigen.^{23,24} In contrast to CD28 expression, which is presented constitutively at high levels on CD4⁺ T cells,¹⁶ only activated T cells express CD40L. It has been shown that blocking antibody to CD40L significantly inhibits TSST-1-induced IL-2, IFN- γ , and TNF- α secretion by activated PBMCs.²⁵ In our experiments disruption of CD40-CD40L interactions by various concentrations of anti-CD40L mAb did not change the steroid responsiveness of superantigen-activated T cells to dexamethasone.

In contrast, our experiments showed that CD28 costimulation is required for the induction of steroid resistance induced by superantigen. Although superantigen stimulation does not increase CD28 expression on T cells, it induces the expression of increased levels of its ligands (CD80 and CD86),²⁵ therefore making superantigen stimulation more dependent from CD28 signaling. We found that enhancement of TCR signaling by CD28-mediated costimulation (by increasing doses of anti-CD28) abrogated the suppressive effects of dexamethasone on anti-CD3-induced proliferation of human PBMCs. Moreover, addition of anti-CD28 to SEB-stimulated T cells in an APC-free system restored T-cell proliferation in the presence of dexamethasone, suggesting that the amount of signaling through CD28 determines the level of steroid resistance after T-cell activation. The anti-CD28 that was used in the current study (clone 37.51, BD Pharmingen) has been used by various investigators as a costimulatory signal for T-cell proliferation.^{26,27} Although the study with CD28 blocking antibody probably will provide more direct evidence for our conclusion, antagonists of CD28 are currently unavailable. On the other hand, the blockade of CD28 ligands (B7.1 and B7.2) might have 2 opposite effects: in addition to reducing T-cell costimulation through CD28, B7 blockade can also prevent CTLA-4 from transmitting a negative-regulatory signal, making it difficult to interpret the results.²⁸ However, the experiments presented do not exclude the possibility that superantigen induces T-cell glucocorticoid resistance by means of a mechanism that does not solely rely on CD28 but is still dependent on some signal from APCs.

Activation of T cells is initiated by formation of an immunologic synapse involving the rearrangement of

receptors into a central supramolecular activation cluster that is enriched with TCRs and CD2 and CD28 molecules.²⁹⁻³¹ The cytoplasmic domain of CD28 has the potential to engage several signaling proteins. It can interact with the SH2 domain of the p85 subunit of PI3K, SH3 domains of IL-2-inducible T-cell kinase and LCK protein tyrosine kinase, and the SH2 domain of growth-factor receptor-bound protein 2.¹⁶

By using various selective protein kinase inhibitors, we examined the potential signaling pathways that are stimulated by anti-CD28 and that lead to augmentation of the CR response. The concentrations of all inhibitors used in this study exceeded their IC₅₀ values. Their ability to completely inhibit phosphorylation of the related substrates after mitogenic stimulation at the concentrations used was confirmed by means of Western blotting. Recruitment of PI3K is considered to be the main signaling pathway that is engaged by CD28.¹⁶ Surprisingly, we found that selective inhibition of PI3K by wortmannin had no effect on superantigen-induced proliferation in the presence of dexamethasone. Previous studies suggested that CD28 can augment signaling from the TCR to the mitogen-activated protein kinase (MAPK) cascade.^{16,17} By using specific MAPK inhibitors at concentrations that were greater than their IC₅₀ values, our goal was to determine which MAPK family member participates in the subversion of corticosteroid response by superantigens. We demonstrate here that signaling through the MEK/ERK pathway is essential for the development of superantigen-mediated resistance to corticosteroids. As assessed with a T-cell proliferation assay, blockade of MEK/ERK signaling with U0126 and PD98059 (MEK/ERK-specific inhibitors) abolished superantigen-induced steroid resistance. In contrast, inhibition of JNK or p38 MAPK did not have such an effect. This provides evidence for a selective role of the MEK/ERK pathway in this process.

Our current data in primary human T cells is consistent with previous reports that activation through the TCR inhibits glucocorticoid-induced apoptosis of mouse T-cell hybridomas and mouse thymocytes.^{32,33} Moreover, this antagonism has been mapped to MAPK/ERK activation.³³ Interestingly, these reported effects on glucocorticoid-induced mouse T-cell apoptosis occurred after activation with anti-CD3 mAbs. In contrast, we found that steroid inhibition of human T-cell proliferation could be induced only by superantigens but not by anti-CD3, despite use of a wide range of anti-CD3 concentrations that exceeded what was used in previous studies of mouse T cells. Although anti-CD3 was able to induce ERK phosphorylation, its ability to do this was transient and weaker than what was observed after superantigen stimulation in human T cells. This difference in strength and duration of ERK phosphorylation appears to account for the ability of superantigen, but not anti-CD3, to sustain corticosteroid resistance in human T cells.

As with all members of the nuclear receptor superfamily, the dimeric GCR mediates transactivation of target genes by binding glucocorticoid response elements in the

promoter region of target genes.⁷ The ligand-activated GCR also interacts with multiple transcription factors and its own coactivators and regulates the function of these signaling molecules (transrepression).³⁴ In addition, the GCR interacts with numerous cytosolic proteins, including chaperones, kinases, phosphatases, nuclear shuttling proteins, and the proteasome. GCR acts as a part of a dynamic network, linking and integrating signaling pathways and regulatory processes in cells.¹⁷ Execution of these various actions begins with nuclear translocation of the GCR after engagement with its ligand. Our experiments indicate that superantigen stimulation results in reduced GCR nuclear translocation, despite the presence of its receptor agonist. Furthermore, we have found that activation of the MEK/ERK pathway is responsible for subversion of this nuclear translocation event.

Many steroid hormone receptors, including the GCR, are phosphoproteins.¹⁸ Evidence to date indicates that steroid hormone receptor phosphorylation serves not as an on-off switch but modulates its function. The GCR is thought to be a substrate for several kinases and phosphatases and has been shown to be polyphosphorylated on serine and threonine residues in the amino-terminal portion of the protein.^{35,36} Although the precise role of each specific phosphorylation event remains unclear, mutation of multiple phosphorylation sites has a profound effect on its ability to translocate to the nucleus, receptor stability, protein half-life, and signaling in a promoter-specific context.³⁶ Several studies suggest a strong cell-cycle dependency of GCR phosphorylation,¹⁸ as well as a potential role of phosphorylation in nuclear cytoplasmic shuttling.^{19,37} In particular, phosphorylation of the GCR controls nuclear translocation of this molecule.¹⁹

A previous study has shown that JNK and ERK activation contributes to the phosphorylation of serine-threonine residues of rat GCR.³⁸ The latter study^{35,38} reported that rat GCR S246 phosphorylation by ERK leads to significant inhibition of the receptor transactivation. By means of sequence comparison of rat and human GCR, it has been suggested that S246 in rat GCR corresponds to S226 in human GCR α . This region represents a highly conserved sequence of GCR between the different species and is likely to be phosphorylated by ERK on the basis of structural homology.³⁸ Recently, it has been reported that enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4⁺ T cells.³⁹ The current study does not exclude the possibility that MEK/ERK signaling can effect the phosphorylation status of the chaperone molecules that are associated with GCR in the cell cytoplasm, which might influence the efficiency of GCR α dissociation from the complex and hence its nuclear translocation in response to steroids. On the other hand, the addition of MEK/ERK inhibitors enhances nuclear translocation of the GCR in superantigen-stimulated PBMCs and therefore restores the phosphorylation status of GCR, its chaperones, or both. Although it is known that p38 MAPK can phosphorylate GCR,⁴⁰ this kinase does not have a role in superantigen-induced steroid resistance because the

presence of the p38 MAPK inhibitor did not enhance cell sensitivity to steroids after SEB stimulation. Data in our current study strongly suggest that ERK phosphorylation of the GCR triggered by superantigens prevents nuclear translocation of the GCR, thereby inducing a state of corticosteroid resistance.

In summary, our study provides direct evidence for the importance of the MEK/ERK signaling pathway in the regulation of human T-cell responsiveness to corticosteroids and a potential role for MEK/ERK inhibitors in the regulation of this process. Our data further strongly suggest that phosphorylation of the GCR is a mechanism for superantigen-induced corticosteroid resistance, and this event identifies GCR phosphorylation sites as a region for further pharmacologic interventions that alter this process.

We thank Maureen Sandoval for her help in preparing this manuscript.

REFERENCES

- Kotzin BL, Leung DY, Kappler J, Marrack P. Superantigens and their potential role in human disease. *Adv Immunol* 1993;54:99-166.
- Leung DY, Meissner HC, Shulman ST, Mason WH, Gerber MA, Glode MP, et al. Prevalence of superantigen-secreting bacteria in patients with Kawasaki disease. *J Pediatr* 2002;140:742-6.
- Paliard X, West SG, Lafferty JA, Clements JR, Kappler JW, Marrack P, et al. Evidence for the effects of a superantigen in rheumatoid arthritis. *Science* 1991;253:325-9.
- Hauk PJ, Wenzel SE, Trumble AE, Szefer SJ, Leung DY. Increased T-cell receptor β 8+ T cells in bronchoalveolar lavage fluid of subjects with poorly controlled asthma: a potential role for microbial superantigens. *J Allergy Clin Immunol* 1999;104:37-45.
- Strickland I, Hauk PJ, Trumble AE, Picker LJ, Leung DY. Evidence for superantigen involvement in skin homing of T cells in atopic dermatitis. *J Invest Dermatol* 1999;112:249-53.
- Leung DY, Travers JB, Giorno R, Norris DA, Skinner R, Aelion J, et al. Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. *J Clin Invest* 1995;96:2106-12.
- Leung DY, Bloom JW. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol* 2003;111:3-22.
- Durongpisitkul K, Gururaj VJ, Park JM, Martin CF. The prevention of coronary artery aneurysm in Kawasaki disease: a meta-analysis on the efficacy of aspirin and immunoglobulin treatment. *Pediatrics* 1995;96:1057-61.
- Leyden JJ, Kligman AM. The case for steroid-antibiotic combinations. *Br J Dermatol* 1977;96:179-87.
- Takei S, Arora YK, Walker SM. Intravenous immunoglobulin contains specific antibodies inhibitory to activation of T cells by staphylococcal toxin superantigens. *J Clin Invest* 1993;91:602-7.
- Schlievert PM, Kelly JA. Clindamycin-induced suppression of toxic-shock syndrome-associated exotoxin production. *J Infect Dis* 1984;149:471.
- Hauk PJ, Hamid QA, Chrousos GP, Leung DY. Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens. *J Allergy Clin Immunol* 2000;105:782-7.
- Goleva E, Kisich KO, Leung DY. A role for STAT5 in the pathogenesis of IL-2-induced glucocorticoid resistance. *J Immunol* 2002;169:5934-40.
- Anderson PS, Geisler C, Buus S, Mariuzza RA, Karjalainen K. The role of the T cell receptor ligand affinity in T cell activation by bacterial superantigens. *J Biol Chem* 2001;276:33452-7.
- Jabara HH, Geha RS. The superantigen toxic shock syndrome toxin-1 induces CD40 ligand expression and modulates IgE isotype switching. *Int Immunol* 1996;8:1503-10.
- Rudd CE, Schneider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol* 2003;3:544-56.

17. Berridge MJ. Lymphocyte activation in health and disease. *Crit Rev Immunol* 1997;17:155-78.
18. Bodwell JE, Webster JC, Jewell CM, Cidlowski JA, Hu JM, Munck A. Glucocorticoid receptor phosphorylation: overview, function and cell cycle-dependence. *J Steroid Biochem Mol Biol* 1998;65:91-9.
19. Wang Z, Frederick J, Garabedian MJ. Deciphering the phosphorylation "code" of the glucocorticoid receptor in vivo. *J Biol Chem* 2002;277:26573-80.
20. Webster JC, Jewell CM, Bodwell JE, Munck A, Sar M, Cidlowski JA. Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. *J Biol Chem* 1997;272:9287-93.
21. Wachs GN, Maibach HI. Co-operative double-blind trial of an anti-biotic/corticoid combination in impetiginized atopic dermatitis. *Br J Dermatol* 1976;95:323-8.
22. Bachert C, Gevaert P, Howarth P, Holtappels G, van Cauwenberge P, Johansson SG. IgE to *Staphylococcus aureus* enterotoxins in serum is related to severity of asthma. *J Allergy Clin Immunol* 2003;111:1131-2.
23. Muraille E, De Smedt T, Thielemans K, Urbain J, Moser M, Leo O. Activation of murine T cells by bacterial superantigens requires B7-mediated costimulation. *Cell Immunol* 1995;162:315-20.
24. Gonzalo JA, Delaney T, Corcoran J, Goodearl A, Gutierrez-Ramos JC, Coyle AJ. Cutting edge: the related molecules CD28 and inducible costimulator deliver both unique and complementary signals required for optimal T cell activation. *J Immunol* 2001;166:1-5.
25. Kum WW, Hung RW, Cameron SB, Chow AW. Temporal sequence and functional implications of V beta-specific T cell receptor down-regulation and costimulatory molecule expression following in vitro stimulation with the staphylococcal superantigen Toxic shock syndrome toxin-1. *J Infect Dis* 2002;185:555-60.
26. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 1992;356:607-9.
27. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995;182:459-65.
28. Haspot F, Villemain F, Laflamme G, Coulon F, Olive D, Tiollier J, et al. Differential effect of CD28 versus B7 blockade on direct pathway of allorecognition and self-restricted responses. *Blood* 2002;99:2228-34.
29. Freiberg BA, Kupfer H, Maslanik W, et al. Staging and resetting T cell activation in SMACs. *Nat Immunol* 2002;3:911-7.
30. Dustin ML, Shaw AS. Costimulation: building an immunological synapse. *Science* 1999;283:649-50.
31. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 1998;395:82-6.
32. Zacharchuk CM, Mercep M, Chakraborti PK, Simons SS Jr, Ashwell JD. Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. *J Immunol* 1990;145:4037-45.
33. Jamieson CA, Yamamoto KR. Crosstalk pathway for inhibition of glucocorticoid-induced apoptosis by T cell receptor signaling. *Proc Natl Acad Sci U S A* 2000;97:7319-24.
34. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev* 2003;24:488-522.
35. Krstic MD, Rogatsky I, Yamamoto KR, Garabedian MJ. Mitogen-activated and cyclin-dependent protein kinases selectively and differentially modulate transcriptional enhancement by the glucocorticoid receptor. *Mol Cell Biol* 1997;17:3947-54.
36. Mason SA, Housley PR. Site-directed mutagenesis of the phosphorylation sites in the mouse glucocorticoid receptor. *J Biol Chem* 1993;268:21501-4.
37. Itoh M, Adachi M, Yasui H, Takekawa M, Tanaka H, Imai K. Nuclear export of glucocorticoid receptor is enhanced by c-Jun N-terminal kinase-mediated phosphorylation. *Mol Endocrinol* 2002;16:2382-92.
38. Rogatsky I, Logan SK, Garabedian MJ. Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase. *Proc Natl Acad Sci U S A* 1998;95:2050-5.
39. Tsioura DC, Rothman PB. Enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4+ T cells. *J Clin Invest* 2004;113:619-27.
40. Irusen E, Matthews JG, Takahashi A, Barnes PJ, Chung KF, Adcock IM. p38 mitogen-activated protein kinase-induced GCR phosphorylation reduces its activity: role in steroid-insensitive asthma. *J Allergy Clin Immunol* 2002;109:649-57.