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Au-ACRAMTU-PEt3 Alters Redox Balance To Inhibit T Cell Proliferation and Function

P. Kent Langston,* Mu Yang, † Ulrich Bierbach, † Derek Parsonage, ‡ Leslie B. Poole, ‡ Madeline J. Price,* and Jason M. Grayson*

Although T cells play a critical role in protection from viruses, bacteria, and tumors (1). Prior to infection, naïve T cells exist in a quiescent, nondividing state (2), relying on oxidative phosphorylation to meet metabolic needs (3). However, during infection, if a naïve T cell encounters a mature dendritic cell presenting cognate Ag, costimulatory molecules, and inflammatory cytokines, it will become activated (4, 5). During this process, a wave of tyrosine phosphorylation and calcium influx occurs that programs new gene expression and drives the cell to enter S phase (6). Following the first division at ~24–48 hours, T cells begin a program of sustained division that allows them to divide ~10–12 times. In addition to elaborating biological functions through clonal expansion, T cells also produce a wide range of cytokines, including IL-2, IL-4, IL-17, TNF-α, and IFN-γ (7). CTLs also use perforin and granzyme-mediated mechanisms to lyse infected cells (1). Following pathogen clearance, effector T cells enter a contraction phase. From 8 to 35 days postinfection (p.i.), Ag-specific T cell numbers decrease 10-fold and the surviving cells differentiate into memory T cells. These cells will be maintained for the life of the animal and can rapidly respond to prevent or lessen the severity of disease upon reinfection.

Although they can perform protective functions during infection and cancer, T cells also cause disease. As part of their normal development, both the B and T cell pools are purged of self-reactive cells through apoptosis (8) and receptor editing (9). Although these mechanisms are highly efficient, they are not perfect, and some self-reactive cells slip through the developmental checkpoints and emigrate to the periphery. Outside the thymus and bone marrow, multiple mechanisms such as regulatory T cells (10), anergy (11), and activation-induced cell death (12) exist to maintain peripheral tolerance. However, for reasons not entirely well understood related to infection, diet, and genetics, tolerance breaks down and autoreactive T cells expand and cause disease. Examples include autoimmune diseases, such as systemic lupus erythematosus (13), rheumatoid arthritis (14), and multiple sclerosis (15), in which immune responses are inappropriately generated against self. In all three of these diseases, self-reactive B and T cells must expand from a low precursor frequency and elaborate effector functions, including cytokine production, for disease to occur. Although self-reactive responses are an important problem, unwanted immune responses during organ transplant and graft-versus-host disease are also major clinical issues. Finally, many individuals suffer from allergies that are unwanted immune responses against innocuous environmental substances (16, 17). Overall, a large portion of clinical disease could be affected if the activation, proliferation, and function of lymphocytes could be precisely controlled.

Multiple drugs, including cyclosporine, FK506, and rapamycin, are available for immune suppression in transplantation and other settings, but they have unwanted side effects, including hypertension and renal nephropathy, that limit their efficacy (18–20). Therefore, other compounds, including cancer drugs such as methotrexate (21) and azathioprine (22), which target rapidly
proliferating cells such as tumors or activated lymphocytes, have been used as immunosuppressive agents with some efficacy. To identify new suppressors of lymphocyte activation, proliferation, and function, we examined the immunosuppressive activity of gold(I) thiourea complexes. Originally developed as treatments for tuberculosis, gold(I) thiourea complexes were found to have efficacy against rheumatoid arthritis. The compounds examined in this study include a series of cationic complexes in which gold is linearly coordinated by the thiourea sulfur of a 9-aminoacridine ligand or structurally related derivatives, and a trans-ligand including (pseudo) halides and phosphines (23). Although these gold-based organometallic complexes had reduced cytotoxicity against NCI-H460, a non–small-cell cancer cell line, they had antituberculosis activity in the submicromolar range. We found that Au-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea (ACRAMTU)–PEt3 is a potent suppressor of murine and human T cell activation. Preincubation with Au-ACRAMTU-PEt3 suppresses the proliferation of CD4+ and CD8+ T cells at a concentration similar to that of commercially available cyclosporine A (CsA). Au-ACRAMTU-PEt3 pretreatment decreases the production of IFN-γ, TNF-α, IL-2, and IL-17 by human and murine CD4+ and CD8+ T cells. When mice were treated with Au-ACRAMTU-PEt3 during viral infection, the expansion of virus-specific CD8+ T cells was decreased 10-fold and viral load was increased. Taken together, these results demonstrate that Au-ACRAMTU-PEt3 has potent immunosuppressive activity and could be used to suppress immune responses during transplantation and autoimmunity.

Materials and Methods

Mice and viral infections
Female 6- to 8-wk-old C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Mice were infected with 2 × 10^5 PFU lymphocytic choriomeningitis virus (LCMV)–Armstrong i.p. and used at the indicated time points. Virus was grown and quantitated as previously described (24). All studies were approved by the Institutional Animal Care and Use Committee of the Wake Forest School of Medicine.

Cell isolation
Spleens were removed from mice following cervical dislocation. After teasing apart the spleen on a wire mesh screen, RBCs were osmotically lysed using ACK Lysis Buffer (Lonza). Splenocytes were resuspended in complete media containing RPMI 1640 supplemented with 10% FCS (HyClone), 1-glutamine, penicillin-streptomycin (Cellgro), nonessential amino acids (Life Technologies), and 2-ME (Life Technologies). Thymocytes were prepared in a similar manner. To isolate bone marrow cells, both femurs were flushed with complete media. RBCs were osmotically lysed as described above, and then cells were washed and counted. Venous blood was drawn from healthy volunteers after approval by the Institutional Review Board. PBMCs were isolated using Lymphoprep media according to the manufacturer’s instructions.

Adoptive transfer and effector CD8+ T cell generation
A naive P14 Thy1.1.PL1 mouse was sacrificed and the spleen was excised. After osmotic lysis, splenocytes were stained with Abs specific for CD8α, CD90.1, and D2GP33-41 MHC class I tetramer. Splenocytes containing 10^4 naive P14 CD8+ T cells were transferred into naive C57BL/6 mice with an engrafment of 10^5 cells (25). Mice were then infected with 2 × 10^5 PFU LCMV strain Armstrong by i.p. injection and were sacrificed on day 8 for effector cell isolation.

Surface and intracellular staining
In this study, the following Abs were used: rat anti-mouse CD8-PerCP, rat anti-mouse CD8-AFC, rat anti-mouse CD4-PerCP, rat anti-mouse CD44-FITC and PE, rat anti-mouse CD90.1–Pacific Blue, rat anti-mouse CD90.1-FITC, rat anti-mouse CD90.1–PE–Cy7, rat anti-mouse CD2L-APC-Cy7, rat anti-mouse IFN-γ–FITC, rat anti-mouse TNF-α–PE, rat anti-mouse IL-2–APC, mouse anti-human IFN-γ–FITC, mouse anti-human TNF-α–PE–Cy7, mouse anti-human IL-17–APC, rat anti-human IL-2–FITC, mouse anti-human CD4–PE, mouse anti-human CD8 APC H7, rabbit anti-phospho-ERK1/2 (Tyr204), and rabbit anti-zAP70 (Thr183/Tyr185). The phospo Abs were purchased from Cell Signaling. All other Abs were purchased from BD Pharmingen. Surface staining was performed by incubating cells in a 1:100 dilution of Ab in 2% FACS buffer (PBS plus 2% FCS) for 30 min on ice. Cells were washed three times with FACS buffer and fixed in 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). After three washes, intracellular cytokine staining was performed using the BD Cytotox/Cytoperm kit (with GolgiPlug and GolgiStop) according to the manufacturer’s protocol. 7-Aminoactinomycin D was added as a surface stain to identify viable cells.

For phospho-ERK1/2 and phospho-zAP70 staining, purified CD8+ T cells were fixed in 2% paraformaldehyde at 37°C for 10 min after stimulation. Samples were then permeabilized with 90% methanol prior to Ab staining according to Cell Signaling’s protocol. Samples were acquired on a BD FACScomp instrument and analyzed using FlowJo software (TreeStar, San Francisco, CA).

5-(and -6)-Chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester oxidation, and MitoSOX labeling
5-(and -6)-Chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester oxidation (DCFDA), was purchased from Invitrogen and resuspended in DMSO as a 2 μM stock. Cells were incubated in a 96-well round-bottom plate with the drug and were loaded with 5 μM DCFDA for 30 min at 37°C in complete media and acquired immediately on a FACScomp instrument. MitoSOX was brought up as 5 μM stock in DMSO, and cells were loaded in 5 μM dye for 30 min in the presence of the drug at 37°C. Data are presented as change in mean fluorescence intensities (MFI) compared with vehicle-treated cells.

T cell purification
Naive CD4+ or CD8+ T cells were negatively selected by magnetic bead enrichment from the spleens of naive C57BL/6 mice, using the Miltenyi MicroBead system according to the manufacturer’s protocol. For human T cell isolation, either the naive CD4+ or CD8+ T cell isolation kit was used. Purity was >95%, as determined by flow cytometry.

CTL line generation
Naive CD8+ T cells were negatively selected by magnetic bead enrichment from the spleens of naive C57BL/6 mice, using the Miltenyi MicroBead system according to the manufacturer’s protocol. A total of 5 × 10^5 purified CD8+ T cells were cultured in media containing 2 μl/ml recombinant murine IL-12 (5) with 10 μg/ml anti-CD3/CD28 stimulation for 2 d. On day 2, cells were removed from Ab-coated wells and allowed to rest overnight before drug treatment and restimulation.

Synthesis of gold(I) complexes
The platinum (26) and gold complexes (23) were synthesized according to published procedures, except for Au-ACRAMTU-PEt3, for which an improved method was developed. To a suspension of 0.097 g (0.25 mmol) ACRAMTU, nitrate salt, in a mixture of 15 ml methanol and 3 ml water was added 0.092 g (0.263 mmol) chlorotriethylphosphine gold(I). After a brief stirring, the suspension turned into a clear solution, and 0.042 g AgCl was removed by syringe filtration, and the solvent was removed by rotary evaporation. The resulting oily material was dried in an oil pump vacuum for 2 h to remove residual water. Dichloromethane (10 ml) was added, and the mixture was stored in the refrigerator for 2 h. Au-ACRAMTU-PEt3 (systematic formula: [Au(ACRAMTU)PEt3(NO3)2] crystallized as a yellow microcrystalline solid, which was recovered by filtration and under vacuum. Yield was 0.135 g (77%).

CFSE labeling
CFSE (Molecular Probes) was resuspended in DMSO as a 5 mM stock and stored at −20°C. Magnetically purified T cells were washed three times in cold PBS and resuspended at a concentration of 2 × 10^6 cells per milliliter in PBS. The CFSE stock was diluted to 6.67 μM in PBS and mixed 1:1 (v/v) with cells, resulting in a final concentration of 3.33 μM CFSE. After 3 min, samples were vortexed and then incubated for an additional 2 min. One-tenth volume of FCS was added, and the cells were vortexed and then incubated for an additional 60 s. The cells were washed three times with complete media and used in experiments.

Proliferation parameter analysis
The division and proliferative indices and the percent divided parameters were calculated using the Proliferation Platform in the FlowJo software package.
Caution flux assay

Fura-Red-acetoxyethyl ester (AM) and Fluo-3-AM were purchased from Molecular Probes and dissolved in DMSO at 1 mM and 1.25 mM stocks, respectively. Magneticnally purified CD8+ T cells were incubated with 5 μM Fura-Red AM and 2.5 μM Flu-3-AM in PBS containing 5% FCS for 30 min at 37˚C in the presence of 0.1% dimethylformamide (DMF) control or Au-ACRAMTU-PEt3. Samples were washed two times with PBS supplemented with 5% FCS and resuspended in the same media containing DMF control or Au-ACRAMTU-PEt3. Cells were coated with biotin-labeled anti-CD3 and anti-CD8α, acquired for 60 s on the FACsCalibur Flow Cytometer, after which 5 μg/ml streptavidin was added to the sample and recording was resumed.

Cell proliferation assay

Drugs were dissolved in DMF as 2 mg/ml stocks. Each drug was then diluted in prewarmed complete medium to make a 20 μg/ml solution. In the rightmost column of a 96-well flat-bottom plate, each 20 μg/ml drug was again diluted 1:3 in the same medium and subsequently diluted across its respective row (from right to left) in a series of 2-fold dilutions. Isotype, vehicle, and rotenone controls were included in the bottom row of each plate. Isotype and vehicle wells received a 0.3% DMF solution, whereas rotenone wells received a 10 μM solution of rotenone in prewarmed complete medium. Magneticnally purified CD4+ or CD8+ T cells from the spleens of naive C57BL/6 mice were then added to the plate and incubated for 60 min at 37˚C. After incubation, the cells were stimulated with plate-bound anti-CD3 (εCD3) and anti-CD28 (εCD28) Abs and returned to the incubator for 72 h. On day 3 poststimulation, Cellitier 96 Aqueous One Solution from Promega was added to each well according to the manufacturer’s protocol. Absorbance was read at 490 nm with a 96-well plate reader every 10 min for 2 h. To calculate the increase in metabolic activity over isotype vehicle-treated cells, the difference between each absorbance and the average isotype absorbance was divided by the difference between the average vehicle and average isotype absorbance. The quotient was then multiplied by 100% to obtain the percent specific increase for each well.

In vitro stimulation

For CD3/CD28 stimulation, 96-well flat-bottom plates were coated with 10 μg/ml anti-CD3 (εCD3) and anti-CD28 (εCD28) overnight at 4˚C. For CD3/CD8 stimulation, purified T cells were coated for 1 min with 5 μg/ml biotin-labeled CD3 and 1 μg/ml biotin-labeled antibody (εCD8α). At the appropriate time point, activation was induced as described by Jia et al. (27) by cross-linking with 5 μg/ml streptavidin.

Au-ACRAMTU-PEt3 treatment

Solid Au-ACRAMTU-PEt3 was dissolved in sterile PBS and given i.p. at 6 mg/kg. Four hours later, mice were infected with LCMV-Armstrong. A maintenance dose was administered to the mice every 12 h for the duration of treatment.

Statistical analysis

Data from vehicle- and Au-ACRAMTU-PEt3-treated mice were analyzed using a two-tailed Student t test. A p value ≤0.05 was considered significant.

Results

Proliferation of naive murine and human CD8+ and CD4+ T cells is inhibited by Au-ACRAMTU-PEt3

To determine whether anticancer transition metal complexes have an inhibitory effect on the activation and proliferation of naive CD8+ T cells, a high-throughput colorimetric cell proliferation assay using a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), was performed. In metabolically active cells, MTS becomes reduced to a soluble formazan product that absorbs at 490 nm. At inhibitory concentrations of drug, wells containing naive murine CD8+ T cells that have been stimulated with plate-bound anti-CD3 (εCD3) and anti-CD28 (εCD28) Abs appear yellow and absorb minimally at 490 nm. Wells containing non-inhibitory concentrations of drug have higher total metabolic activity associated with cell growth and proliferation, turning the media a dark brown color and absorbing strongly at 490 nm. Fig. 1A demonstrates the concentration-dependent effect of several anticancer drugs on T cell proliferation. The Journal of Immunology 3

Phosphorylation of ZAP70 is a key early step in T cell activation that increases the kinase activity of the protein. Intracellular staining revealed that there was an ~1.2-fold increase in the MFI of ZAP70 phosphorylation within 2 min of CD3 and CD8 stimulation (Fig. 2A). Treatment of cells with 274 nM Au-ACRAMTU-PEt3 did not decrease phosphorylation. H2O2 was used as a positive control for ZAP70 phosphorylation because it decreases total phosphatase activity. Flux of Ca2+ through the CRAC (calcium release–activated calcium) channel is another early process critical for T cell activation. To determine whether Au-ACRAMTU-PEt3 prevents T cell proliferation by interfering with Ca2+ flux, magnetically purified naive CD8+ T cells were incubated with both Au-ACRAMTU-PEt3 and the Ca2+-specific dyes Fura-Red-AM and Fluo-3-AM. Cells incubated with a 0.3% DMF control had a basal level of Ca2+, which rapidly increased following incubation.
with biotin-labeled anti-CD3 and anti-CD8α, followed by streptavidin crosslinking; however, cells treated with Au-ACRAMTU-PEt3 exhibited decreased Ca2+ flux following stimulation (Fig. 2B). We next examined the effect of Au-ACRAMTU-PEt3 incubation on MAPK signaling. Incubation with biotin-labeled isotype Abs elicited phospho-ERK1/2 staining that was indistinguishable from its isotype
control (Fig. 2C). After 5 min of CD3/CD8 stimulation, the MFI of phospho-ERK1/2 staining increased ∼1.6-fold in vehicle-treated cells, but in cells treated with Au-ACRAMTU-PEt3 there was no change in staining.

Incubation with Au-ACRAMTU-PEt3 disrupts the mitochondrial thioredoxin–thioredoxin reductase system and oxidizes CD8+ T cells

Previous studies have identified mitochondrial thioredoxin reductase II (TrxR2) as a potential target of Et3PAu(I) complexes (28). To determine the effect of Au-ACRAMTU-PEt3 on the function of TrxR2, naive splenocytes and magnetically purified CD8+ T cells were treated with vehicle, 4.36 μM Au-ACRAMTU-PEt3, 10 mM H2O2, or 20 mM DTT. Following precipitation of proteins and incubation of sonicated cells in 15 mM 4-acetoamido-4′-maleimidylstilbene-2,2′-disulfonic acid for 3 h, proteins were separated on a 15% polyacrylamide separating gel, transferred to a nitrocellulose membrane, and probed for thioredoxin II (Trx2) using a polyclonal IgG anti-Trx2 Ab. Splenocytes treated with vehicle had approximately a 1:1 ratio of reduced to oxidized Trx2; however, Au-ACRAMTU-PEt3-treated splenocytes had primarily the oxidized form of Trx2 (Supplemental Fig. 2A). The difference in the oxidation state of Trx2 in vehicle- and Au-ACRAMTU-PEt3–treated CD8+ T cells was even more pronounced. This finding demonstrates that incubation with Au-ACRAMTU-PEt3 interferes with the Trx2–TrxR2 redox system, causing a shift from reduced to oxidized Trx2 in naive CD8+ T cells.

Because we observed changes in the oxidation of mitochondrial thioredoxin, we measured whether steady-state levels of reactive oxygen intermediates were altered following Au-ACRAMTU-PEt3 incubation. Short-term (30 min) incubation with 274 nM Au-ACRAMTU-PEt3 modestly, but reproducibly, increased the MFI (Fig. 2D) of DCFDA, which measures cellular peroxides, and MitoSOX, which allows the levels of mitochondrial superoxide to be determined. Representative primary data are presented in Supplemental Fig. 2B and 2C. To determine whether the shift in intracellular redox balance blocks activation, naive CD8+ T cells were pretreated with NAC, Au-ACRAMTU-PEt3, or NAC, and Au-ACRAMTU-PEt3 and ERK1/2 staining was performed as described above. Four mice were examined in three independent experiments.

FIGURE 2. Au-ACRAMTU-PEt3 incubation inhibits calcium flux and ERK1/2 phosphorylation during CD8+ T cell activation. (A) Magnetic microbead–purified CD8+ T cells were incubated with 0.1% DMF control or Au-ACRAMTU-PEt3 for 30 min. After washing, cells were incubated with biotin-labeled anti-CD3 and anti-CD8 Abs or isotype control. Cross-linking was induced by incubation with streptavidin for 2 min, followed by intracellular staining with isotype or anti–phospho-ZAP70 Abs. Numbers in the upper right-hand corners indicate the stimulated and unstimulated MFI. The H2O2 plot is included as a positive control. (B) Purified CD8+ T cells were incubated with Fura-Red-AM and Fluo-3-AM in the presence of Au-ACRAMTU-PEt3 for 30 min. After washing, cells were resuspended in PBS supplemented with FCS, incubated with biotin-labeled anti-CD3 and anti-CD8 Abs, and were acquired on a cytometer for 1 min prior to incubation with streptavidin to induce crosslinking. Acquisition was resumed for an additional 5 min and 30 s. The ratio of Fluo-3 to Fura-Red fluorescence was recorded as a function of time. (C) For MAPK measurement, cells were harvested after 5 min of stimulation and stained with anti–phospho-ERK1/2 Abs. (D) Naive CD8+ T cells were treated with Au-ACRAMTU-PEt3 and labeled with either DCFDA or MitoSOX for 30 min and acquired. The increase in MFI compared with 0.1% DMF control–treated samples is plotted. Three mice were examined in three independent experiments. *Significant difference between vehicle- and Au-ACRAMTU-PEt3–treated cells, p < 0.05. (E) Naive CD8+ T cells were treated with media or 5 mM NAC for 1 h followed by treatment with media or 274 nM Au-ACRAMTU-PEt3 for 1 h at 37˚C. Cells were then activated with CD3 and CD28 Abs. Proliferation was assessed by loss of CFSE fluorescence after activation. Representative plots are shown for three mice in two independent experiments. (F) CD8+ T cells were pretreated with NAC, Au-ACRAMTU-PEt3, or NAC, and Au-ACRAMTU-PEt3 and ERK1/2 staining was performed as described above. Four mice were examined in three independent experiments.

control (Fig. 2C). After 5 min of CD3/CD8 stimulation, the MFI of phospho-ERK1/2 staining increased ∼1.6-fold in vehicle-treated cells, but in cells treated with Au-ACRAMTU-PEt3 there was no change in staining.

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5 mM NAC modestly inhibited proliferation (Supplemental Fig. 2D–H); however, as in our prior results, incubation with 274 nM Au-ACRAMTU-PEt3 prevented proliferation (Fig. 2E). When naive CD8+ T cells were pretreated with 5 mM NAC and subsequently incubated with 274 nM Au-ACRAMTU-PEt3, activation and proliferation were restored to the level observed with NAC alone (Fig. 2E, Supplemental Fig. 2). To determine the molecular mechanism by which NAC rescues the proliferative block of Au-ACRAMTU-PEt3, we repeated our signal transduction analysis. Incubation of T cells with NAC and Au-ACRAMTU-PEt3 failed to rescue the block in Ca2+ flux, ERK1/2 phosphorylation, and activation, we next sought to determine if Au-ACRAMTU-PEt3 could affect CD8+ T cell function. Splenocytes containing 10^5 naive P14 CD90.1+ TCR transgenic CD8+ T cells were transferred into naive C57BL/6 mice, which were subsequently infected with 2 \times 10^3 PFU of LCMV-Armstrong. To measure effector T cell function, mice were sacrificed on day 8 p.i., and intracellular cytokine staining was performed for IFN-γ, TNF-α, and IL-2 following pretreatment with vehicle or Au-ACRAMTU-PEt3 and stimulation with GP33-41 peptide. Because CD107a and b are co-stimulatory molecules, we next sought to determine whether Au-ACRAMTU-PEt3 is capable of inhibiting human CD8+ T cell function, intracellular cytokine staining was performed for IFN-γ, TNF-α, IL-2, IL-17, and IL-4 following pretreatment with vehicle or Au-ACRAMTU-PEt3 and stimulation with PMA/ION in vitro. Incubation with Au-ACRAMTU-PEt3 significantly reduced the percentage of human CD8+ T cells that produced IFN-γ, TNF-α, IL-2, and IL-17 following PMA/ION stimulation (Fig. 4D, 4F) but increased the percentage of CD4+ T cells that produced IL-4 (Fig. 4H), compared with vehicle-treated cells. Modulation of CD4+ T cell cytokine production resulting from incubation with Au-ACRAMTU-PEt3 was concentration dependent (Fig. 4G, 4I). Thus, Au-ACRAMTU-PEt3 incubation significantly decreases production of IFN-γ, TNF-α, IL-2, and IL-17 by CD4+ T cells while increasing production of IL-4.

The expansion of CD8+ T cells is reduced in Au-ACRAMTU-PEt3–treated mice following acute infection

Owing to the profound effect of Au-ACRAMTU-PEt3 incubation on effector CD8+ T cell function, we next sought to determine if Au-ACRAMTU-PEt3 incubation could also modulate CD4+ T cell cytokine production. Naïve C57BL/6 mice were infected with 2 \times 10^3 PFU of LCMV-Armstrong. To measure T cell function, mice were sacrificed on day 8 p.i., and intracellular cytokine staining was performed for IFN-γ, TNF-α, and IL-2 following pretreatment with vehicle or Au-ACRAMTU-PEt3 and stimulation with GP61–80 peptide in vitro. The percentage of CD4+ T cells that produced cytokines following GP61–80 stimulation was significantly reduced by Au-ACRAMTU-PEt3 in a concentration-dependent manner (Fig. 4A–C). To determine whether Au-ACRAMTU-PEt3 is also capable of inhibiting human CD4+ T cell function, intracellular cytokine staining was performed for IFN-γ, TNF-α, IL-2, IL-17, and IL-4 following pretreatment with vehicle or Au-ACRAMTU-PEt3 and stimulation with PMA/ION in vitro. Incubation with Au-ACRAMTU-PEt3 significantly reduced the percentage of human CD4+ T cells that produced IFN-γ, TNF-α, IL-2, and IL-17 following PMA/ION stimulation (Fig. 4D, 4F) but increased the percentage of CD4+ T cells that produced IL-4 (Fig. 4H), compared with vehicle-treated cells. Modulation of CD4+ T cell cytokine production resulting from incubation with Au-ACRAMTU-PEt3 was concentration dependent (Fig. 4G, 4I). Thus, Au-ACRAMTU-PEt3 incubation significantly decreases production of IFN-γ, TNF-α, IL-2, and IL-17 by CD4+ T cells while increasing production of IL-4.

The expansion of CD8+ T cells is reduced in Au-ACRAMTU-PEt3–treated mice following acute infection

Owing to the profound effect of Au-ACRAMTU-PEt3 incubation on T cell activation, proliferation, and function in vitro, we hypothesized that treating C57BL/6 mice with this drug during the expansion phase following an acute viral infection would significantly decrease the T cell response. Naïve C57BL/6 mice were treated with 6 mg/kg of Au-ACRAMTU-PEt3 every 12 h for 8 d, at which point the mice were sacrificed and the spleen, lymph nodes, bone marrow, and thymus were removed. Administering Au-ACRAMTU-PEt3 resulted in a 10-fold reduction in the total number of CD8+CD4+ double positive T cells in the thymus, whereas the total number of CD8+ and CD4+ single positive T cells in the thymus, spleen, lymph nodes, and bone marrow (Fig. 6A–C).
were minimally affected. To test the hypothesis that Au-ACRAMTU-PEt3 treatment would decrease CD8+ T cell expansion following acute viral infection, splenocytes containing 10^5 naive P14 CD8+ T cells were transferred into naive C57BL/6 mice, which were subsequently infected with LCMV-Armstrong. On day 8 p.i., mice were sacrificed, the spleen was removed, splenocytes were treated with vehicle or Au-ACRAMTU-PEt3 for 1 h at 37°C, and stimulated (A) with GP33-41 peptide and labeled (E and F) with anti-CD107a/b Abs for 5 h at 37°C. Following stimulation, cells were stained with anti-CD8, anti-CD90.1, IFN-γ, TNF-α, and IL-2 Abs. Dot plots (A) are gated on CD90.1+ CD8+ T cells, and the number in the plots indicates the percentage of CD90.1+ CD8+ T cells that are present in that quadrant. The percentage of CD8+ CD90.1+ T cells that produced IFN-γ, TNF-α, and IL-2 (B) or CD107a and b (F) was determined. The mean and SD are plotted. Five to six mice were analyzed in a minimum of two independent experiments. PBMCs were isolated from healthy human donors and were then treated with vehicle or Au-ACRAMTU-PEt3 for 1 h at 37°C, followed by stimulation (C) with PMA/ION for 5 h at 37°C. Following stimulation, cells were stained with CD8, IFN-γ, TNF-α, and IL-2 Abs. Dot plots (C) are gated on CD8+ T cells, and the number in the plots indicates the percentage of CD8+ T cells that are present in that quadrant. (D) The percentage of drug-treated CD8+ T cells that produced IFN-γ, TNF-α, and IL-2, compared with vehicle-treated CD8+ T cells, was determined. The mean percent of vehicle cytokine production and SD are plotted. Four subjects were analyzed in four independent experiments. *Significant difference between vehicle- and Au-ACRAMTU-PEt3–treated cells, p ≤ 0.05.

**FIGURE 3.** Incubation with Au-ACRAMTU-PEt3 inhibits murine and human effector T cell function. C57BL/6 mice were injected i.v. with 10^5 Thy1.1+ (CD90.1+) P14 cells and were subsequently infected with 2 × 10^3 PFU of LCMV-Armstrong. On day 8 p.i., mice were sacrificed, the spleen was removed, splenocytes were treated with vehicle or Au-ACRAMTU-PEt3 for 1 h at 37°C, and stimulated (A) with GP33-41 peptide and labeled (E and F) with anti-CD107a/b Abs for 5 h at 37°C. Following stimulation, cells were stained with anti-CD8, anti-CD90.1, IFN-γ, TNF-α, and IL-2 Abs. Dot plots (A) are gated on CD90.1+ CD8+ T cells, and the number in the plots indicates the percentage of CD90.1+ CD8+ T cells that are present in that quadrant. The percentage of CD8+ CD90.1+ T cells that produced IFN-γ, TNF-α, and IL-2 (B) or CD107a and b (F) was determined. The mean and SD are plotted. Five to six mice were analyzed in a minimum of two independent experiments. PBMCs were isolated from healthy human donors and were then treated with vehicle or Au-ACRAMTU-PEt3 for 1 h at 37°C, followed by stimulation (C) with PMA/ION for 5 h at 37°C. Following stimulation, cells were stained with CD8, IFN-γ, TNF-α, and IL-2 Abs. Dot plots (C) are gated on CD8+ T cells, and the number in the plots indicates the percentage of CD8+ T cells that are present in that quadrant. (D) The percentage of drug-treated CD8+ T cells that produced IFN-γ, TNF-α, and IL-2, compared with vehicle-treated CD8+ T cells, was determined. The mean percent of vehicle cytokine production and SD are plotted. Four subjects were analyzed in four independent experiments. *Significant difference between vehicle- and Au-ACRAMTU-PEt3–treated cells, p ≤ 0.05.

**Discussion**

In this study, we have examined the effect of Au-ACRAMTU-PEt3 treatment on T cell activation, proliferation, and function. In this article, we report five novel observations. First, preincubation of murine and human T cells with Au-ACRAMTU-PEt3 prevented activation and proliferation following stimulation with CD3 and CD28 Abs. Second, Au-ACRAMTU-PEt3 incubation resulted in decreased calcium flux and ERK1/2 phosphorylation in CD8+ T cells. Third, Au-ACRAMTU-PEt3 incubation shifts the redox...
balance in cells to a more oxidized state, and its antiproliferative effects can be rescued by antioxidant treatment. Fourth, Au-ACRAMTU-PEt3 incubation significantly decreased CD4+ and CD8+ T cell cytokine production in vitro in a concentration-dependent manner. Fifth, the expansion of rapidly proliferating effector CD8+ T cells following an acute viral infection was significantly suppressed by twice-daily treatment with Au-ACRAMTU-PEt3. Taken together, these results demonstrate the efficacy of Au-ACRAMTU-PEt3 as an inhibitor of T lymphocyte activation, proliferation, and function.

What are the mechanisms by which Au-ACRAMTU-PEt3 inhibits T cell activation and proliferation? On the basis of our in vitro studies, T cell activation is inhibited at two early steps: calcium flux and ERK1/2 phosphorylation. In addition, we demonstrate that Au-ACRAMTU-PEt3 pretreatment increases the steady-state level of reactive oxygen species (ROS) in the mitochondria. In addition to playing a critical role in energy production, mitochondria are a major source of ROS during T cell activation (29). Following anti-CD3 or peptide stimulation, ROS, including superoxide and hydrogen peroxide, are produced by both CD4+ and CD8+ T cells (30, 31). This production is essential, as blocking it with antioxidants decreases the expansion of T and B cells in vitro and in vivo (32, 33). Following ROS production in T and B cells, cysteine becomes reversibly oxidized to sulfenic acid (31, 34). Combined with our observations that Au-ACRAMTU-PEt3 induces a shift from reduced to oxidized thioredoxin, these results are consistent with a model in which drug addition induces mitochondrial oxidative stress resulting in altered signal transduction. Further support for this idea is provided by the observation that addition of NAC, an antioxidant, is able to protect against the antiproliferative effects of Au-ACRAMTU-PEt3 by restoring ERK2 phosphorylation. Importantly, two recent studies have demonstrated that ERK2 can undergo sulfenic acid modification (35, 36) that could inhibit its activation. Although ERK1 is dispensable for peripheral T cell activation and proliferation, ERK2 function is required to increase Bcl-xL mRNA levels while suppressing those of Bim (37). Loss of ERK2 function leads to decreased T cell survival that can be rescued by loss of Bim.
addition to direct oxidative modification of ERK1/2, it is possible that upstream regulators could become oxidized in Au-ACRAMTU-PEt3–treated cells. Gorelik and colleagues (38) demonstrated that oxidative modification of protein kinase C contributed to ERK inactivation observed in lupus T cells. A similar mechanism could be contributing to the effects of Au-ACRAMTU-PEt3. Importantly, the antiproliferative effects of Au-ACRAMTU-PEt3 were not restricted to in vitro experiments, as they were also observed in rapidly dividing double negative T cells in the thymus and effector CD8+ T cells in the spleen during viral infection. In summary, these

FIGURE 5. Au-ACRAMTU-PEt3 treatment inhibits CD8+ T cell cytokine production in an autonomous manner. Splenocytes were isolated from naive C57BL/6 mice, and CD8+ T cells were purified by magnetic microbeads. Naive CD8+ T cells were stimulated with plate-bound CD3 and CD28 Abs in the presence of 2 U/ml recombinant murine IL-12 for 2 d. Cells were removed from Ab-coated plates and allowed to rest overnight and were then pretreated for 1 h at 37°C with the indicated concentration of Au-ACRAMTU-PEt3, followed by PMA and ION stimulation for 5 h. Afterward, cells were intracellularly stained for their production of IFN-γ, TNF-α, and IL-2 (A). The number in the plots indicates the percentage of CD8+ T cells that are present in that quadrant. For IFN-γ (B), TNF-α (C), and IL-2 (D), the percentage of cells positive for each cytokine relative to vehicle-treated samples was determined. In addition, the MFI of each cytokine was compared with vehicle-treated samples. The mean percent of vehicle cytokine production and SD are plotted. Four lines were analyzed in three independent experiments. *Significant difference between vehicle- and Au-ACRAMTU-PEt3–treated cells, \( p \leq 0.05 \).
findings demonstrate that Au-ACRAMTU-PEt3 increases oxidative stress to decrease lymphocyte proliferation in vitro and in vivo.

In addition to decreasing proliferation, Au-ACRAMTU-PEt3 also inhibits T cell cytokine production and degranulation. By what mechanism does Au-ACRAMTU-PEt3 modulate T cell function? Using intracellular cytokine staining, we found that effector T cells exhibited a major decrease in cytokine production at nanomolar concentrations of Au-ACRAMTU-PEt3. Again, similar to proliferation, we hypothesize that Au-ACRAMTU-PEt3 alters redox homeostasis, inhibiting cytokine production. Of note, IL-2 production was more sensitive to Au-ACRAMTU-PEt3 incubation than was IFN-γ or TNF-α. This result is consistent with our observations of decreased calcium flux, as transcription of the IL-2 gene requires activation of the calcineurin-NFAT pathway following TCR stimulation (39). In addition to inhibiting cytokine production, Au-ACRAMTU-PEt3 treatment also inhibited cytolytic activity of effector CD8+ T cells. In contrast to the complete shutoff in cytokine production, Au-ACRAMTU-PEt3 inhibited degranulation by 50%. Further support for the altered redox balance hypothesis is provided by increased production of IL-4 by CD4+ T cells following Au-ACRAMTU-PEt3 preincubation. Because dead cells are excluded from the analysis, this argues against toxicity decreasing all cytokine production, and instead supports our model of redox regulation, as prior reports have demonstrated that oxidizers such as mercuric chloride are able to induce IL-4 transcription in mast cells (40). Interestingly, PBMCs and splenocytes required a higher concentration of Au-ACRAMTU-PEt3 to inhibit cytokine production than did purified effector CD8+ T cells. One potential explanation could be that heterogeneous mixtures such as PBMCs and splenocytes contain other cells that modulate the redox environment. Prior studies have shown that dendritic cells and monocytes modulate the extracellular redox environment to affect T cell proliferation and function (41). It is possible that the presence of these cells and their secretion of thioredoxin could alter the redox balance in T cells, requiring more Au-ACRAMTU-PEt3 to inhibit signaling.

In conclusion, our studies demonstrate that the gold(I) complex Au-ACRAMTU-PEt3 is a potent inhibitor of T cell activation, proliferation, and function in vitro and in vivo. We have also advanced the current understanding of the mode of action of gold thiourea complexes by demonstrating that, in addition to interfering with the Trx2-TrxR2 redox system, Au-ACRAMTU-PEt3 inhibits Ca^{2+} flux and ERK1/2 phosphorylation. Furthermore, we observed that twice-daily i.p. injections of 6 mg/kg of Au-ACRAMTU-PEt3 were sufficient to decrease the effector CD8+ T cell response to an acute viral infection 10-fold—a level of inhibition equivalent to that achieved with daily i.p. injections of 60 mg/kg of pharmaceutical-grade CsA (data not shown). Togather, our observations in vitro and in vivo suggest that Au-ACRAMTU-PEt3 may be used as a potential treatment for autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis and for preventing rejection of transplanted tissues. Furthermore, they highlight the key role that redox balance plays in the activation, proliferation and function of T cells. Finally, the mechanism of action of these mixed-ligand phosphine/thiourea complexes at the molecular level remains to be established. Although the TrxR targeted [AuPEt3] moiety appears to be a prerequisite for potent activity in vitro and in vivo, the role of the acridinylthiourea as a carrier ligand and its fate in circulation remain elusive. Thus, one goal of this drug development program will be to establish structure–activity relationships in libraries of structurally diverse derivatives.
Figure 1. Incubation with Au-ACRAMTU-PET3 decreases the division and proliferative indexes and the percentage of naïve murine and human T cells that divide. Splenocytes were isolated from naïve C57BL/6 mice, and CD8+ T cells (A) or CD4+ T cells (B) were purified by magnetic microbeads and the number of viable cells was determined on day 3 anti-CD3/CD28 stimulation in the presence of increasing Au-ACRAMTU-PET3. The division index, proliferation index, and % divided were determined for murine CD8+ (C) and CD4+ T cells (D). Similar measurements were made for human CD8+ (E) and CD4+ (F) T cells on day 5 anti-CD3/CD28 stimulation in the presence of increasing Au-ACRAMTU-PET3. Four subjects were used in 4 independent experiments. The mean and SD are plotted. *, significant difference between vehicle- and Au-ACRAMTU-PET3-treated cells, p<0.05.
Figure 2. Au-ACRAMTU-PET₃ modulates the redox status of cells. (A) Splenocytes and purified CD8⁺ T cells (5x10⁶) from naive C57BL/6 mice were treated for 30 min in 10 mM H₂O₂ or 20 mM dithiothreitol (DTT) or for 60 min in 4.36 μM Au-ACRAMTU-PET₃ or vehicle. Proteins were then precipitated and alkylated with AMS prior to separation on a 15% polyacrylamide separating gel. This blot is representative of Trx2 oxidation state in the presence of H₂O₂, DTT, Au-ACRAMTU-PET₃, or vehicle in 2 independent experiments. CD8⁺ T cells were purified from naive C57BL/6 mice and then were preincubated with vehicle or 274 nM Au-ACRAMTU-PET₃ for 60 minutes. Cells were then incubated with either (B) DCF-DA or (C) MitoSox for 30 minutes, washed and then acquired immediately. The open histogram represents vehicle-treated cells, while filled histograms are from Au-ACRAMTU-PET₃ pretreated cells. Staining is representative of 3 mice in two independent experiments. Magnetic microbead purified CD8⁺ T cells were incubated with 0.1% DMF control or 5mM NAC for 60 minutes at 37°C. Afterwards cells were incubated with 0.1% DMF or 274 nM Au-ACRAMTU-PET₃ for 60 minutes. Cells were then placed on αCD3/αCD28 coated plates and the number of viable cells (D), percentage of viable cells (E), division index (F), proliferation index (G), and % divided (H) were determined on day 3. Four mice were analyzed in 3 independent experiments. The mean and standard deviation are plotted. *, significant difference between vehicle- and Au-ACRAMTU-PET₃ treated cells, p<0.05, student’s t test.
Figure 3. Quantification of T cell signal transduction. (A) Magnetic microbead purified CD8+ T cells were incubated with with 0.1% DMF or 274 nM Au-ACRAMTU-PEt3 for 60 minutes. Cells were then coated with αCD3/αCD8 antibodies followed by streptavidin crosslinking. The peak ratio of Fluo-3/Fura-Red was determined for 3 mice in 2 independent experiments. The average and standard deviation are plotted. Alternatively, magnetic microbead purified CD8+ T cells were incubated with 0.1% DMF control or 5mM NAC for 60 minutes at 37°C. Afterwards cells were incubated with 0.1% DMF or 274 nM Au-ACRAMTU-PEt3 for 60 minutes. Cells were then coated with αCD3/αCD8 antibodies followed by streptavidin crosslinking. Intracellular staining for (B) phospho-Zap-70 or (C) phospho-ERK1/2 was performed. The percent increase in m.f.i. relative to the vehicle-treated sample was calculated. The mean and standard deviation are plotted. *, significant difference between vehicle- and Au-ACRAMTU-PEt3-treated cells, p<0.05, student’s t test.
Figure 4. Quantification of cell viability following incubation with Au-ACRAMTU-PEt. (A) PBMCs were incubated with 0.1% DMF or the indicated concentration of Au-ACRAMTU-PEt₃ for 60 minutes. Cells were then stimulated with PMA/ION in the presence of GolgiPlug and GolgiStop for 5 hours. Following surface staining cells were incubated with 7-AAD, washed and then intracellular staining for cytokines was performed. The percent of cells that were 7AAD⁺ was determined for (A) PBMCs, (B) CD8⁺ or (C) CD4⁺ T cells. The mean and standard deviation are plotted. *, significant difference between vehicle- and Au-ACRAMTU-PEt₃⁺ treated cells, p<0.05, student's t test.