The anti-cancer drug, phenoxodiol, kills primary myeloid and lymphoid leukemic blasts and rapidly proliferating T cells

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ABSTRACT

Background
The redox-active isoflavene anti-cancer drug, phenoxodiol, has previously been shown to inhibit plasma membrane electron transport and cell proliferation and promote apoptosis in a range of cancer cell lines and in anti-CD3/anti-CD28-activated murine splenocytes but not in non-transformed WI-38 cells and human umbilical vein endothelial cells.

Design and Methods
We determined the effects of phenoxodiol on plasma membrane electron transport, MTT responses and viability of activated and resting human T cells. In addition, we evaluated the effect of phenoxodiol on the viability of leukemic cell lines and primary myeloid and lymphoid leukemic blasts.

Results
We demonstrated that phenoxodiol inhibited plasma membrane electron transport and cell proliferation (IC50 46 µM and 5.4 µM, respectively) and promoted apoptosis of rapidly proliferating human T cells but did not affect resting T cells. Phenoxodiol also induced apoptosis in T cells stimulated in HLA-mismatched allogeneic mixed lymphocyte reactions. Conversely, non-proliferating T cells in the mixed lymphocyte reaction remained viable and could be restimulated in a third party mixed lymphocyte reaction, in the absence of phenoxodiol. In addition, we demonstrated that leukemic blasts from patients with primary acute myeloid leukemia (n=22) and acute lymphocytic leukemia (n=8) were sensitive to phenoxodiol. The lymphocytic leukemic blasts were more sensitive than the myeloid leukemic blasts to 10 µM phenoxodiol exposure for 24h (viability of 23±4% and 64±5%, respectively, p=0.0002).

Conclusions
The ability of phenoxodiol to kill rapidly proliferating lymphocytes makes this drug a promising candidate for the treatment of pathologically-activated lymphocytes such as those in acute lymphoid leukemia, or diseases driven by T-cell proliferation such as autoimmune diseases and graft-versus-host disease.

Key words: phenoxodiol, anti-leukemic, proliferating T cells, auto-immune disease, graft-versus-host disease.

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Introduction

The redox active synthetic isoflavene, phenoxodiol, is currently being studied in phase III clinical trials for drug-resistant ovarian cancer, and in early stage clinical trials for prostate and cervical cancer, both as a single agent and as an adjunct to chemotherapy.1,2 Phenoxodiol has been referred to as a pan-cancer drug,3 causing apoptosis in cancer cell lines and murine cancer models via both intrinsic and extrinsic pathways.2,4,14 Plasma membrane electron transport (PMET) has been shown to be a target of phenoxodiol in several studies with cancer cell lines.3,5,15

PMET consists of an outer membrane (surface) NADH-oxidoreductase, redox-recycling plasma membrane ubiquinone and an inner membrane NADH-oxidoreductase.16 PMET plays a vital role in maintaining both plasma membrane and intracellular redox balances to support continued cell survival, function and growth of fast proliferating cells.15,17,18 Most cancer cells actively increase glucose uptake to meet increased energy demands; this is associated with increased NADH recycling to maintain intracellular NADH/NAD+ ratios. Cells that generate most of their energy through oxidative phosphorylation recycle NADH mainly through mitochondrial electron transport. In contrast, glycolytic cells recycle NADH via lactate dehydrogenase and PMET.1,15

Although cancer cells exhibit elevated PMET, several orders of magnitude higher than their non-proliferative counterparts,18 different types of cancer cells rely on PMET for NADH recycling to different extents, depending on their level of glycolytic metabolism.21 Nevertheless, PMET provides an excellent target for anticancer drug development, especially in the treatment of highly refractory glycolytic cancers.16

Binding of phenoxodiol to a recombinant truncated form of a tumor-specific surface oxidoreductase (tNOX) has been proposed as a primary mechanism of action of phenoxodiol in cancer cells as well as being responsible for its cancer specificity.5 However, we have previously reported that phenoxodiol inhibited PMET and cell proliferation,17 and induced apoptosis in proliferating murine splenocytes, suggesting that phenoxodiol may also target non-malignant proliferating lymphocytes.

In this study, we examined the effects of phenoxodiol on rapidly proliferating and resting human T cell in vitro and in mixed lymphocyte reactions (MLR). In addition, we investigated the effect of phenoxodiol on leukemic cell lines and primary leukemic blasts isolated from the bone marrow of patients with acute myeloid leukemia (AML) or acute lymphocytic leukemia (ALL).

Design and Methods

Materials

Bags of concentrated blood (50 mL) from healthy volunteers were provided by the Red Cross Blood Bank (Melbourne, Australia). Bone marrow samples were obtained from the tissue bank at the Peter MacCallum Cancer Institute after obtaining ethical approval from the Peter MacCallum Tissue Research Management Committee (project number 07/14).

The ALL-derived MOLT-4, multiple myeloma-derived U-226 and multiple myeloma-derived RPMI-8226 cell lines were obtained from the cell culture collection at the Peter MacCallum Cancer Centre. The AML-derived HL-60 and HL-60p cell lines were obtained from the cell culture collection at the Malaghan Institute. HL-60p cells are mitochondrial gene knockout mutants, derived from parental HL-60 cells by long-term culture (6-12 weeks) with 50 ng/mL filter-sterilized ethidium bromide.22 These cells are unable to perform mitochondrial electron transport and rely on glycolysis for ATP production. The absence of mitochondrial DNA was verified by polymerase chain reaction.

2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium monosodium salt (WST-1) and 1-methoxyphenazine methylsulfate (1mPMS) were purchased from Dojindo Laboratories (Kumamoto, Japan). Phenoxodiol was obtained from Novogen Inc (NSW, Australia). Human anti-CD3 and anti-CD28, mouse anti-human anti-CD3 (APC), anti-CD19 (PECy7), anti-CD14 (APC-Cy7), anti-CD15 (PE), annexin V (FITC) (AV) and propidium iodide (PI) were from Pharmingen (Becton Dickinson, North Ryde, Australia). Human recombinant interleukin-2 was obtained from the Biological Resources Branch Preclinical Repository, NCI (Frederick, MD, USA). Unless otherwise stated all other reagents were from Sigma (St. Louis, Missouri, USA). Phenoxodiol was stored in solid form under nitrogen gas to prevent oxidation and dissolved prior to each experiment in dimethyl-sulfoxide (DMSO) at 10000x or 1000x the final concentration before being diluted in Hanks balanced salts solution (HBSS) or RPMI-1640 medium (GIBCO-BRL, Grand Island, NY, USA), supplemented with 10% (v/v) fetal calf serum (FCS). Control experiments were conducted using DMSO at the same concentrations as those used in the phenoxodiol treatment experiments.

Cell lines were grown in RPMI-1640 medium (GIBCO-BRL) supplemented with 5% (v/v) FCS, 2 mM glutamate, 25 µg/mL penicillin, 25 µg/mL streptomycin, 50 µg/mL uridine and 1 mM pyruvate to densities of 1-2×10^6 cells/mL (exponential stage), at 37°C in 5% CO2 in a humidified incubator.

Collection, isolation and storage of peripheral blood mononuclear cells from buffy coats

Peripheral blood mononuclear cells (PBMC) were isolated fromuffy coats of blood bags from healthy volunteers using Ficoll gradients. PBMC were resuspended in RPMI-1640 medium (GIBCO-BRL) supplemented with 10% (v/v) FCS and viable cells were counted by trypan blue exclusion. PBMC were stored at 20-30×10^6 cells/vial in 1.5 mL 90% FCS+10% DMSO in liquid nitrogen. The frequency of different cell types in the PBMC samples used for these experiments was determined by FACS analysis to be as follows: 70-80% CD3+ (T lymphocytes), 5-15% CD19+ (B lymphocytes), 10-20% CD14+ (monocytes), 5-10% CD56+ (NK cells), <5% CD15+ (neutrophils). The CD5+ cell population consisted of 30-40% CD8+ and 60-70% CD4+ T cells.
In vitro activation of T cells

Immediately prior to experiments, frozen PBMC were thawed, centrifuged at 1400 rpm in a Multifuge 3s (Heraeus) for 4 min, washed in RPMI + 10% FCS and resuspended in T-cell medium (RPMI + 10% FCS + 10 µM 2-mercaptoethanol (Sigma), 1 x glutamine, 1 x non-essential amino acids (Gibco)) at 2x10^6 cells/mL. Cells (2x10^6) were activated by adding 10 µg/mL anti-CD3 and 5 µg/mL anti-CD28 and 20 U/mL interleukin-2 per well. Cells were incubated at 37°C in 5% CO2 in a humidified incubator for 4 to 5 days until large numbers of large spherical aggregates or bursts of lymphocytes were observed. Activated proliferating T cells were pooled from the wells for further experiments. Resting T cells were incubated for the same period of time but in the presence of 20 U/mL interleukin-2 only.

Mixed lymphocyte reactions

Mixed lymphocyte reactions (MLR) were established using unrelated, HLA-mismatched healthy donor blood samples as donor/recipient pairs. Stimulator PBMC were γ-irradiated with 30 Gy as described previously.29 Responder PBMC were washed twice in PBS, 5,6-carboxy-succinimidyl-fluorescein-ester (CSFE)-labeled (1.25 µM, Sigma) at room temperature for 5 min, and washed twice in PBS/10% FCS. Two hundred thousand stimulator and responder cells were added per well at a 1:1 ratio in a 96 U-well plate in T-cell medium. Stimulator and responder cells were also plated alone, and all cells were cultured in 5% CO2, at 37°C in a humidified incubator. Viability, CSFE fluorescence and cell surface antigen expression were determined by flow cytometry. Cells were washed twice in FACS buffer (PBS, 2% FCS), resuspended in 50 µL/5x10^6 cells and analyzed on a Becton Dickinson LSRII FACS analyzer using FlowJo (TreeStar) software.

Isolation of leukemic blasts from bone marrow samples of leukemia patients

Tissue banked bone marrow samples from patients with AML (n=22) and ALL (n=8), containing more than 80% blasts, were thawed, centrifuged at 1400 rpm in a Multifuge 3s (Heraeus) for 4 min, washed twice in RPMI and resuspended in RPMI + 10% FCS. Aliquots were incubated for 24h in the presence of 0.1% DMSO (control) or 10 mM phenoxodiol and examined for morphology and viability. Percentage apoptosis was determined by AV/PI staining as [% viable blasts after phenoxodiol treatment] / [% viable blasts in control sample].

Plasma membrane electron transport activity measured by WST-1/PMS reduction

WST-1/PMS reduction rates were measured in a microplate format as described previously.20 Briefly, exponentially growing cells were centrifuged at 1400 rpm in a Multifuge 3s (Heraeus) for 4 min, washed and resuspended in HBSS buffer. For each assay, 50 µL of a 2x10^6 cells/mL cell suspension were pipetted into flat-bottomed microplate wells containing 50 µL of the inhibitor-buffer solution, resulting in a final concentration of 1x10^6 cells/mL. Following 48 h incubation, dye reduction was initiated by adding 10 µL of 5 mg/mL MTT to each well. After 2 h, 100 µL of lysing buffer were added and formazan crystals dissolved by manual pipetting using a multichannel pipette before measuring A570 in a BMG FLUOstar OPTIMA plate reader.

Cell proliferation and metabolic activity of resting T cells measured by MTT reduction

MTT reduction, which is an indicator of cellular metabolic activity, was measured as previously described21 in a microplate format as follows: exponentially growing cells or resting T cells were centrifuged at 1400 rpm in a Multifuge 3s (Heraeus) for 4 min, washed and resuspended in HBSS buffer. For each assay, 50 µL of a 2x10^6 cells/mL cell suspension were pipetted into flat-bottomed microplate wells containing 50 µL of the inhibitor buffer solution, resulting in a final concentration of 1x10^6 cells/mL. Following 48 h incubation, dye reduction was initiated by adding 10 µL of 5 mg/mL MTT to each well. After 2 h, 100 µL of lysing buffer were added and formazan crystals dissolved by manual pipetting using a multichannel pipette before measuring A570 in a BMG FLUOstar OPTIMA plate reader.

Cell viability measured by trypan blue exclusion

Proliferating and resting T cells were centrifuged at 1400 rpm in a Multifuge 3s (Heraeus) for 4 min at room temperature, resuspended in fresh T-cell medium in 96 U-well plates (200 µL per well at densities of 2x10^6 cells/mL) in the presence of phenoxodiol or 0.1% DMSO and incubated in 5% CO2 at 37°C in a humidified incubator. Viable cells, as determined by trypan blue exclusion, were counted in a Neubauer hemocytometer every 24 h for several days.

Statistical analysis

A paired two-tailed Student’s t test was used to determine the statistical significance of the difference between the viability of blasts from patients with AML (n=22) and ALL (n=8). A p value of less than 0.05 was considered to be statistically significant.

Results

Phenoxodiol inhibits plasma membrane electron transport, cell proliferation and viability of proliferating human T cells

We investigated the effect of phenoxodiol on PMET (Figure 1A) by measuring reduction of the cell impermeable tetrazolium dye, WST-1, in the presence of its obli- gate intermediate electron acceptor, 1mPMS.18 Exposure to phenoxodiol for 30 min inhibited PMET of proliferating T cells (IC50 of 46 µM) but had only a minor inhibitory effect on resting T cells (IC50 >200 µM). The effect of 48 h phenoxodiol exposure on T-cell proliferation and metabolic activity of resting T cells (Figure 1B) was determined by measuring the reduction of the tetrazolium salt, MTT. In contrast to WST-1, MTT is reduced intracellularly by reducing equivalents such as NADH produced during glycolysis; the level of MTT reduction is therefore directly related to the level of metabolic activity.21 MTT
reduction was inhibited in the presence of phenoxodiol in proliferating T cells (IC50=5.4 μM), but not in resting T cells (IC50>200 μM). Using anti-CD3, anti-CD28 and interleukin-2, proliferating T cells had a cycling time of 21 h. Resting T cells did not proliferate but remained viable, regardless of the presence of 10 μM phenoxodiol (Figure 1C). However, the viability of proliferating T cells was severely compromised by exposure to phenoxodiol.

**Brief exposure to phenoxodiol is enough to kill proliferating human T cells**

We next investigated whether a short exposure to phenoxodiol was sufficient to induce T-cell apoptosis. Rapidly proliferating T cells were treated with 10 μM phenoxodiol for different periods of time, washed twice and incubated for a further 24 h in fresh T-cell medium. A brief 1 min exposure to 10 μM phenoxodiol induced apoptosis in proliferating T cells to the same extent as was seen after a 24 h exposure. Pre-incubation of phenoxodiol in 90% FCS or 90% human serum did not affect the activity of phenoxodiol to kill proliferating T cells (Table 1), showing that the activity of phenoxodiol is not affected by serum proteins.

**Phenoxodiol eliminates proliferating responder T cells in HLA-mismatched mixed lymphocyte reactions**

We further tested the ability of phenoxodiol to induce apoptosis in proliferating T cells in an HLA-mismatched MLR. CFSE-labeled responder cells were mixed with HLA-mismatched γ-irradiated stimulator cells in the presence of 0.1% DMSO (control) and 10 μM phenoxodiol on day 0. We analyzed responder cells for proliferation (CFSE) and viability (AV) at day 8. Strong activation and proliferation of allogeneic T cells (CD3+CFSE+AV) population was seen in the control MLR (46.5%, Figure 2B) but not in the phenoxodiol-treated MLR (2%, Figure 2D). In contrast, a resting responder cell population (CD3+CFSE+AV) remained present in both control and phenoxodiol-treated MLR.

**Unstimulated T cells respond to foreign antigen after transient exposure to phenoxodiol**

We next determined whether a transient exposure to phenoxodiol affected the ability of unstimulated T cells to respond normally to foreign antigen. Resting T cells were incubated with 10 μM phenoxodiol for 24 h, washed twice and stimulated for 3 days with a third party antigen in complete T-cell medium. After 3 days, CD3+CFSE+AV populations were similar in both control MLR (62.6%) and phenoxodiol-treated MLR (46.5%) (Figure 3). Transient exposure of unstimulated T cells to phenoxodiol did not, therefore, inhibit these cells' ability to be subsequently activated.

**Resting responder T cells can be restimulated in a third party mixed lymphocyte reaction**

We extended the previous experiment by determining the effect of phenoxodiol exposure on responder T cells that had previously been exposed to, but not activated in, a MLR. We set up two consecutive sets of MLR, mixing responder T cells and γ-irradiated stimulator cells on day 0, adding 10 μM phenoxodiol or 0.1% DMSO on day 5, and analyzing proliferation and viability of responder T cells.
cells on day 8. The viable resting (CD3+CFSEhiAV–) populations were sorted by FACS and stimulated in a subsequent third party MLR in the absence of phenoxodiol. Previously resting T cells proliferated (CD3+CSFEloAV– population in Figure 4D) in response to third party stimulation, suggesting that prior exposure to phenoxodiol does not affect either the viability or the functionality of non-proliferating T cells.

**Phenoxodiol causes apoptosis in leukemic cell lines and leukemic blasts from bone marrow samples**

We have previously shown that phenoxodiol inhibited PMET and induced apoptosis in AML-derived HL-60 and HL60ρ0 cells,15 T-cell ALL-derived MOLT-4 cells and B-cell ALL-derived CEM cells (unpublished results). Here, we examined phenoxodiol-induced apoptosis in a panel of hematologic cancer cell lines as well as in primary leukemic blasts from bone marrow samples of ALL and AML patients (Figure 5). We were unable to establish the effect of phenoxodiol on PMET in these primary leukemic blasts, because of the low numbers of cells in the clinical samples. The characteristics of these clinical samples are described in Online Supplementary Table S1. The sensitivity to phenoxodiol varied between cell lines and primary cells. ALL blasts, in keeping with the discussed phenoxodiol sensitivity of normal proliferating lymphocytes, were more consistent in their response to phenoxodiol and significantly more sensitive than AML blasts (p=0.0002).

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**Figure 2.** Exposure to phenoxodiol eliminates proliferating responder T cells in HLA-mismatched MLR. Responder PBMC cells were exposed to HLA-mismatched γ-irradiated stimulator cells in the presence of 0.1% DMSO (A and B) and 10 µM phenoxodiol (C and D) on day 0 and analyzed on day 8. A. and C. are scatter plots of control and phenoxodiol-treated responder PBMC, respectively. Gates indicate the CD3+ T lymphocyte populations. B. and D. are proliferation plots of the corresponding CD3+ T lymphocyte population, showing viable resting populations (CSFE AV–) in both control and phenoxodiol-treated MLR and viable proliferating populations (CFSE AV–) in the control MLR only. Results are representative of three separate experiments.

**Figure 3.** Transient exposure of unstimulated T cells to phenoxodiol does not affect their ability to respond in HLA-mismatched MLR. Responder cells were pre-incubated with 0.1% DMSO (control) or 10 µM phenoxodiol for 24 h, washed twice in fresh medium, mixed with HLA-mismatched γ-irradiated stimulator cells on day 0 and analyzed by FACS on day 8. See Figure 2 for an explanation of plots. Results are representative of two separate experiments.

**Figure 4.** Transient exposure of resting responder T cells to phenoxodiol does not affect their ability to respond in a subsequent HLA-mismatched third party MLR. Viable resting responder T cells (CD3+CSFEAV–) were sorted on day 8 of an HLA-mismatched MLR and either incubated for a further 8 days (A and B) or restimulated in a second subsequent third party MLR, with HLA-mismatched γ-irradiated stimulator cells from a third person (C and D). Only restimulated responder T cells showed strong proliferation in the subsequent MLR (CSFE+ population in D but not in B). Results are representative of two separate experiments.
Discussion

This paper describes, for the first time, the effect of phenoxodiol on primary leukemic cells and extends previous findings that phenoxodiol kills rapidly proliferating mouse T cells. The sensitivity of proliferating human T cells to phenoxodiol, as demonstrated by the IC50 values for inhibition of PMET (46 μM) and cell proliferation (5.4 μM) are within the range previously described for HL-60 cells (32 μM and 2.3 μM, respectively) and HL-60p2 cells (70 μM and 6.7 μM, respectively) and appear to reflect differences in cell cycle times (15 h, 21 h and 29 h for HL-60 cells, proliferating T cells and HL-60p2 cells, respectively). The almost 10-fold difference in IC50 values between the two assays can be explained by the fact that PMET activity was measured 30 min after exposure to phenoxodiol and metabolic activity 48 h after phenoxodiol exposure. Apoptosis measurements demonstrated that a 1 min exposure to 10 μM phenoxodiol resulted in the same level of apoptosis in proliferating T cells after 24 h incubation, compared with the level caused by continuous 24 h exposure.

Although PMET may be a target for phenoxodiol, the link between inhibition of PMET, inhibition of cell proliferation and induction of apoptosis is not clear. The viability experiments (Figures 1 and 2) demonstrated that a brief exposure to 10 μM phenoxodiol induced apoptosis in T cells that were rapidly proliferating, but had little effect on the viability and proliferating capacity of resting T cells. However, the lack of a CFSE population in cells exposed to phenoxodiol in the MLR (Figure 3D) may not be entirely due to apoptosis of activated T cells. At this stage we cannot exclude the possibility that phenoxodiol, in addition to inducing apoptosis, inhibits activation of resting T cells; this needs to be further explored in the future.

The clear lack of effect of phenoxodiol on resting T cells indicates that proliferating T cells may possess a phenoxodiol-sensitive site that is absent in resting T cells. Furthermore, binding of phenoxodiol to this site induces irreversible effects on the viability of rapidly dividing T cells.

Phenoxodiol has been shown to bind to and inhibit the activity of a recombinant tumor-specific NADH-oxidase (tNOX) from HeLa cells, thought to be involved in PMET. DeLuca et al. proposed that inhibition of PMET via tNOX results in increased levels of intracellular NADH and membrane ubiquinol, inhibiting sphenosine kinase and increasing membrane ceramide levels, which induce G1 arrest and apoptosis. In contrast, activity of constitutively-expressed cell surface NADH-oxidase, CNOX, present on both cancer and non-cancer cells, was said to be unaffected by phenoxodiol; thus, phenoxodiol has been heralded as a "pan-cancer drug." However, our results in rapidly proliferating mouse and human T cells do not support the view that phenoxodiol exclusively affects malignant cells. Other authors have also questioned the cancer-specificity of phenoxodiol, because of its potent anti-angiogenic properties, inhibiting endothelial cell proliferation, migration and capillary formation, and its neurotoxic effect at doses of 10 μM. Molecular studies in cancer cell lines have shown that phenoxodiol causes proteosomal degradation of the anti-apoptotic protein XIAP activating the extrinsic apoptotic pathway. Phenoxodiol was also shown to activate the intrinsic apoptotic pathway by caspase-2-dependent activation of the pro-apoptotic protein, Bid, amplified by Bax. The effect of phenoxodiol on expression of these molecular markers in proliferating and resting T cells is the subject of ongoing work.

Although phenoxodiol effectively induces apoptosis in different types of malignant cells as well as in rapidly proliferating T cells, it has little effect on the viability of proliferating human umbilical vein endothelial cells. WI38 human fibroblasts and other proliferating non-transformed human cell lines. In addition, phenoxodiol was shown to be well-tolerated by patients in early stage clinical trials, indicating that not all dividing cells are sensitive to phenoxodiol. The differential effect of phenoxodiol on induction of apoptosis in primary leukemic cells, with ALL blasts being significantly more sensitive to phenoxodiol than AML blasts, is pertinent in this respect and also points to a certain amount of specificity of phenoxodiol for pathologically activated lymphocytes.

The concentrations of phenoxodiol used in this study (10 μM in most experiments) are easily reached in patients. A stage I clinical trial by Choueri et al. demonstrated that phenoxodiol could be administered safely as a 7-day continuous infusion given every 2 weeks up to a
dose of 27.0 mg/kg/day. This gave maximum blood serum levels of 60 µg/mL (250 µM), with a mean value of 37 µg/mL (130 µM) which was reached after 24 h infusion. The results presented here show that, in addition to its effect on solid cancers, phenoxodiol induces apoptosis in primary acute myeloid and lymphoid leukemic cells and rapidly proliferating T cells. These findings suggest that phenoxodiol may be effective in anti-leukemic therapy or in diseases driven by T-cell activation such as autoimmune diseases and graft-versus-host disease. The combination of potency and specificity of phenoxodiol against proliferating T cells suggests that this drug may be able to limit graft-versus-host disease whilst maintaining non-proliferating T-cell populations responsible for post-transplant immunological reconstitution.

Authorship and Disclosures

PMH: responsible for most of the experimental design, execution and analysis of most of the experimental work, and writing the manuscript; JED: responsible for the experimental design, execution and analysis of the mixed lymphocyte reactions and contributed to writing and editing the manuscript; PN: substantial input into experimental design, evaluation and analysis, and contributed to writing and editing the manuscript.

The authors reported no potential conflicts of interest.