

Original articles

# Differential expression of genes encoding immunomodulatory proteins in response to amphotericin B in human mononuclear cells identified by cDNA microarray analysis

P. David Rogers<sup>1-3\*</sup>, Margaret M. Pearson<sup>2</sup>, John D. Cleary<sup>1,2</sup>, Donna C. Sullivan<sup>2,3</sup> and Stanley W. Chapman<sup>2,3</sup>

<sup>1</sup>Department of Pharmacy Practice, Division of Infectious Diseases; Departments of <sup>2</sup>Medicine and

<sup>3</sup>Microbiology, University of Mississippi Schools of Pharmacy and Medicine, Jackson, MS 39216-4505, USA

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**Amphotericin B (AMB) is an antifungal agent that possesses immunomodulatory properties that may contribute to its infusion-related toxicity and activity. It has previously been shown to induce the expression of genes encoding the cytokines interleukin (IL)-1 $\beta$  and tumour necrosis factor (TNF)- $\alpha$  and the chemokines IL-8 and macrophage inflammatory protein (MIP)-1 $\beta$  in the human monocytic cell line THP-1. In an effort to identify additional AMB-responsive genes, the gene expression profiles of both THP-1 cells and human peripheral blood mononuclear cells (hPBMCs) on exposure to AMB were assessed using cDNA microarray analysis. In addition to genes known to be AMB responsive, we found the genes encoding IL-1 $\alpha$  and MIP-1 $\alpha$  to be AMB responsive in both THP-1 cells and hPBMCs. Increases in MIP-1 $\alpha$  and MIP-1 $\beta$  were also observed in the supernatants of hPBMCs exposed to AMB. The expression of several genes in response to AMB was unique to either cell type. Furthermore, variability in gene expression in hPBMCs was observed between donors. These genes and respective gene products may have significance in the infusion-related toxicity and activity of AMB.**

## Introduction

Amphotericin B (AMB) is a polyene antifungal antibiotic with activity against a number of pathogenic fungi.<sup>1,2</sup> In addition to its direct antifungal effects, it has diverse immunomodulatory properties. It inhibits chemotactic responsiveness and diminishes phagocytic capacity and killing in human neutrophils.<sup>3,4</sup> In human lymphocytes, it inhibits both spontaneous and antigen-induced lymphocyte transformation as well as antibody-dependent cellular toxicity.<sup>5</sup> Natural killer (NK) cell activity has also been reported to be impaired.<sup>6,7</sup> AMB also increases the production of interleukin (IL)-1 $\beta$  and tumour necrosis factor (TNF)- $\alpha$  in human and murine mononuclear cells.<sup>8–10</sup>

AMB-associated infusion-related adverse effects, such as fever and chills, occur in up to 70% of patients receiving the

drug and have been correlated with serum concentrations of IL-1 $\beta$  and TNF- $\alpha$ .<sup>10,11</sup> Furthermore, it has been postulated that the immunomodulatory properties of AMB may also contribute to its antifungal activity. Recently, we have shown that AMB activates the genes encoding IL-1 $\beta$ , TNF- $\alpha$ , IL-1Ra, macrophage inflammatory protein (MIP)-1 $\beta$ , monocyte chemotactic protein (MCP)-1, IL-8 and intercellular adhesion molecule (ICAM)-1 in the human monocytic cell line THP-1. Activation of these genes correlated with increased production of their respective gene products in these cells.<sup>12–15</sup>

THP-1 cells have proven to be useful as a model for understanding the biology of monocytic cells. However, it has been suggested that the use of human peripheral blood mononuclear cells (hPBMCs) would be more representative of the *in vivo* response to AMB than human cell lines.<sup>16</sup> In an effort

\*Correspondence address. Department of Clinical Pharmacy, College of Pharmacy, University of Tennessee, 26 South Dunlap Street, Memphis, TN 38163, USA. Tel: +1-901-448-1493; Fax: +1-901-448-1741; E-mail: drogers@utmem.edu

to expand our initial findings in THP-1 cells, studies were undertaken to identify additional genes encoding immunomodulatory proteins that are AMB responsive in THP-1 cells and hPBMCs *in vitro*. By using hPBMCs from several donors, we also wished to identify genes with expression that may vary between subjects. Such genes may represent a pharmacogenomic basis for identifying patients who may either experience toxicity or gain an added benefit from the immunomodulatory properties of AMB.

## Materials and methods

### Reagents and cell culture

RPMI 1640 medium and AMB were obtained from Sigma (St Louis, MO, USA). Low-endotoxin fetal bovine serum was purchased from Summit Biotech (Fort Collins, CO, USA) and Life Technologies (Grand Island, NY, USA). The human mononuclear cell line THP-1 (ATCC TIB 202) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 20  $\mu$ M  $\beta$ -mercaptoethanol, 2  $\mu$ M L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (supplemented medium). The cells were cultured in suspension at 37°C and 5% CO<sub>2</sub> in a humidified incubator and were maintained at 0.1–2.0  $\times 10^6$  cells/mL, passing 2–3 times weekly as needed to maintain viability. Cells were pelleted and resuspended with fresh medium in T-75 tissue culture flasks (Sarstedt, Newton, NC, USA) at a concentration of 10<sup>6</sup> cells/mL and allowed to equilibrate for 24 h before experimental use, to avoid any confounding gene expression that might result from handling.

For isolation of hPBMCs, fresh blood was extracted from healthy volunteers ( $n = 5$ ), layered over an equal volume of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 350g for 30 min at room temperature. The mononuclear cell layer was aspirated, washed twice in large volumes of sterile RPMI 1640 medium at 600g for 10 min at 4°C and resuspended at a concentration of 10<sup>6</sup> cells/mL in supplemented medium. All reagents used in cell culture, including AMB, were either purchased as being lipopolysaccharide (LPS)-free or assayed for LPS contamination by the gel clot method utilizing the *Limulus* amoebocyte lysate (LAL) assay (Associates of Cape Cod, Woods Hole, MA, USA) as described previously.<sup>12</sup>

AMB concentrations of >10  $\mu$ g/mL have been associated with low cell viability.<sup>9</sup> Therefore, a concentration of 5  $\mu$ g/mL was used in this study. Cells were exposed to AMB for 2 h, at which point total RNA was isolated. This 2 h time-point is representative of the window in which AMB-associated infusion-related toxicity is observed clinically.<sup>2</sup>

Cell viability for each experimental condition was assessed by erythrosin B exclusion. Samples of 200  $\mu$ L were removed from each well at the end of the exposure and combined with 20  $\mu$ L of erythrosin B (4 mg/mL). A 10  $\mu$ L volume from this

mixture was then examined in a haemocytometer under a standard microscope. Viable and non-viable cells within a 1 mm<sup>2</sup> area were then counted. Cells that excluded the dye were considered viable. Cell viability did not differ between the conditions studied.

### Probe preparation

Total RNA was isolated with the Trizol reagent (Life Technologies) according to the manufacturer's instructions. A 1–10  $\mu$ g sample of total RNA in DEPC-treated H<sub>2</sub>O was mixed with 2  $\mu$ g of oligo(dT) primer (10–20-mer mixture; ResGen, Huntsville, AL, USA), heat denatured at 70°C for 10 min in a regulated water bath then chilled on ice. This was added to a mixture of 0.1 M DTT (Life Technologies); 20 mM dATP, dGTP, dATP (Amersham Pharmacia Biotech, Piscataway, NJ, USA); 300 U of Superscript II reverse transcriptase (BRL/Life Technologies); 100  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Pharmacia Biotech); in a total volume of 24  $\mu$ L and incubated at 37°C for 90 min in a regulated water bath. On completion of the reverse transcription step, 70  $\mu$ L of DEPC-treated H<sub>2</sub>O was added and the mixture was placed on ice. Probes were purified on Bio-6 Chromatography columns (Bio-Rad, Hercules, CA, USA) and placed on ice.

### Microarray hybridization

All hybridizations were performed using a GF211 'Known Genes' Genefilter cDNA array (ResGen). This array consists of >4000 individual elements, each representing a known human gene (see <http://www.resgen.com/product/MammGF.php3> for a complete list of genes represented on this array). Each element consists of a 0.5 ng spot of cDNA spotted on to the nitrocellulose membrane. The cDNAs, ~1 kb in length, contain the entire 3'UTR and are all sequence-verified clones.

Filters were initially pre-washed in 0.5% SDS (heated until boiling) for 5 min with agitation. Pre-hybridization of the array with 5  $\mu$ g of poly(dA) (ResGen) and 5  $\mu$ g of heat-denatured human Cot-1 DNA (Life Technologies) in 5 mL of Microhyb solution (ResGen) was performed in a roller bottle for 2 h in a hybridization oven at 42°C. Labelled probe was denatured for 3 min by boiling in water and then placed immediately into the hybridization mixture and incubated for an additional 18 h at 42°C. After 18 h, the hybridization mixture was discarded and the array was washed twice in 2 $\times$  SSC, 1% SDS for 20 min at 50°C with continued rotation in the roller oven. A third wash was performed in 0.5 $\times$  SSC, 1% SDS for 15 min at 55°C. After the third wash, the filter was placed on moistened Whatman paper and covered with Saran wrap. The array was then placed in an imager cassette with a phosphorimager storage screen (Molecular Dynamics, Sunnyvale, CA, USA) and exposed for 2–5 days.

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### Image analysis

Exposed phosphorimager storage screens were scanned once on a Storm 860 Phosphorimager (Molecular Dynamics) at a resolution of 50  $\mu\text{m}$  and visualized using ImageQuant NT 4.2a (Molecular Dynamics). Images were imported into Pathways 3.0 (ResGen) for analysis according to the internal software protocol. Mapping of individual elements to an internal reference database was achieved by aligning the images on to a software-based matrix using a total of 16 control elements representing total genomic control DNA. Results were normalized to the average of all data points for each array.

### Microarray data analysis

Software-based pair-wise comparisons of the normalized images were made between the images obtained from hybridization of labelled mRNA taken from cells exposed to AMB and those exposed to medium alone. Changes in expression levels were calculated using normalized intensities and given as ratios and were visualized by generating false-colour images that displayed differences in transcript levels as a variation in colour intensity. Filters were stripped after each hybridization by washing them in 0.5% SDS (heated until boiling) for 1 h with constant agitation. Stripping efficiency was assessed using a Geiger counter.

For THP-1 cells, genes were considered AMB-responsive if they were found to be up- or down-regulated at least two-fold in each individual experiment. For hPBMCs, genes were required to be up- or down-regulated at least two-fold in cells from at least two of the five donors, and by a normalized average of two-fold or greater in response to AMB compared with cells exposed to medium alone.

### Cytokine and chemokine measurements

IL-1 $\beta$ , MIP-1 $\alpha$  and MIP-1 $\beta$  concentrations were determined with commercial ELISA kits (R&D Systems, Minneapolis,

MN, USA). After exposure to either AMB 5  $\mu\text{g/mL}$  or medium alone for 6 h, particulates were removed from cell cultures by centrifugation. Supernatants were stored at  $-70^\circ\text{C}$  until assay. Experiments and all assays were performed in duplicate. Absorbances were read at the appropriate wavelength on a Dynex Technologies MRX microplate reader (Dynex Technologies, Chantilly, VA, USA).

## Results

### *AMB activates genes encoding immunomodulatory proteins in THP-1 cells*

THP-1 cells were exposed to AMB 5  $\mu\text{g/mL}$  for 2 h. This time-point was chosen because it represents the period in which AMB-induced infusion-related toxicity is observed. It is also an ideal time-point for the identification of primary gene expression responses, as opposed to those with expression secondary to the initial response. Of the 4325 genes evaluated, 11 were found to be differentially expressed in response to a 2 h exposure to AMB (Table 1). These included two genes previously identified as being AMB responsive in THP-1 cells: those encoding MIP-1 $\beta$  and IL-8. The remainder represent genes newly found to be AMB responsive in the present study. It should be noted that the gene encoding TNF- $\alpha$  was not represented on the array. Furthermore, the average expression ratio for the gene encoding IL-1 $\beta$  (1.96) fell just below the predetermined threshold of a two-fold change in expression.

### *AMB activates genes encoding immunomodulatory proteins in hPBMCs*

hPBMCs from five healthy volunteers were collected and assessed for differential gene expression in response to a 2 h exposure to AMB 5  $\mu\text{g/mL}$ . By evaluating cells from multiple donors, we hoped to identify genes that are consistently differentially expressed in response to AMB and those with

**Table 1.** Genes found to be differentially expressed in response to exposure to AMB 5  $\mu\text{g/mL}$  for 2 h in THP-1 cells

Accession no.	Title	Gene	Ratio (average)
H62864	small inducible cytokine A4 (MIP-1 $\beta$ )	<i>SCYA4</i>	20.34
W92764	TNFAIP6	<i>TNFAIP6</i>	3.58
AA476272	TNFAIP3	<i>TNFAIP3</i>	3.54
W56300	nuclear factor of $\kappa$ light polypeptide gene enhancer in B-cells inhibitor- $\alpha$	<i>NFKBIA</i>	3.39
AA102526	IL-8	<i>IL8</i>	3.17
AA936768	IL-1 $\alpha$	<i>IL1A</i>	2.61
W96155	v-jun avian sarcoma virus 17 oncogene homologue	<i>JUN</i>	2.2
AA677522	small inducible cytokine A3 (homologous to mouse MIP-1 $\alpha$ )	<i>SCYA3</i>	2.09
AA773894	zinc finger protein 43 (HTF6)	<i>ZNF43</i>	0.45
H59861	thrombomodulin	<i>THBD</i>	0.44
AA464600	v-myc avian myelocytomatosis viral oncogene homologue	<i>MYC</i>	0.4

**Table 2.** Expression ratios for genes found to be differentially expressed in response to exposure to 5 µg/mL AMB for 2 h in hPBMCs

Accession No.	Title	Gene	Fold expression (donor)					Average expression
			1	2	3	4	5	
H62864	small inducible cytokine A4 (MIP-1β)	<i>SCYA4</i>	35.87	8.76	5.71	2.35	4.32	11.402
AA102526	IL-8	<i>IL8</i>	17.01	3.03	1.51	0.8	1.56	4.782
AA677522	small inducible cytokine A3 (MIP-1α)	<i>SCYA3</i>	3.82	3.05	4.52	2.14	4.14	3.534
AA045326	protein tyrosine phosphatase receptor	<i>PTPRJ</i>	1.39	0.98	1.04	9.1	2.47	2.996
T72877	IL-1 receptor antagonist	<i>IL1RN</i>	3.82	3.05	3.02	1.86	1.9	2.73
AA936768	IL-1α	<i>IL1A</i>	2.13	3.34	2.16	1.76	3.05	2.488
AA150507	IL-1β	<i>IL1B</i>	3.04	2.92	2.57	1.36	1.44	2.266
AA629692	chaperonin containing TCP1(subunit 5)	<i>CCT5</i>	2.45	3.02	2.15	1.45	2.09	2.232
AA775874	ribosomal protein L18	<i>RPL18</i>	2.82	2.49	1.36	1.57	2.86	2.22
AA644211	COX-2	<i>PTGS2</i>	2.12	2.13	2.35	1.92	2.47	2.198
R87497	G6PD	<i>G6PD</i>	1.37	1.55	1.2	3.39	2.78	2.058
AA156571	alanyl-tRNA synthetase	<i>AARS</i>	2.09	1.23	1.18	1.57	4.18	2.05
AA418410	cyclophilin	<i>USA-CYP</i>	1.95	1.09	1.06	3.34	2.74	2.036
R71691	TNF receptor-associated factor 1	<i>TRAF1</i>	2.19	1.5	1.13	2	3.31	2.026
AA599175	nuclease-sensitive element binding protein 1	<i>NSEPI</i>	1.48	1.1	0.49	2.6	4.37	2.008
AA425861	enoyl coenzyme A hydratase 1	<i>ECH1</i>	1.74	0.8	0.78	3	3.68	2
AA630800	interferon γ-inducible protein 30	<i>IFI30</i>	0.54	0.2	0.08	0.31	1.26	0.478

expression that may vary between donors. Seventeen genes were found to be differentially expressed in response to AMB (Table 2). Genes that were up-regulated and have been found to be AMB responsive in THP-1 cells in the present study or in previous studies included those encoding MIP-1α, MIP-1β, IL-1α, IL-1β, IL-1Ra and IL-8. The remainder represents genes uniquely found to be AMB responsive in hPBMCs.

Included in Table 2 are the fold changes in gene expression in hPBMCs from the five individual donors in response to AMB exposure. Only two AMB-responsive genes were found to have expression ratios ≥2-fold that observed with cells exposed to medium alone in all five donors: the genes encoding MIP-1α and MIP-1β. Genes with expression ratios of ≥1.5-fold those of their respective controls in all five donors included those encoding IL-1Ra, IL-1α and cyclooxygenase (COX)-2. AMB-induced expression of the gene encoding IL-1β was ≥2-fold that observed with medium alone in only three subjects. The two subjects who failed to produce an IL-1β response were the only subjects whose hPBMCs produced ≥2-fold changes in expression of glucose-6-phosphate dehydrogenase (G6PD) in response to AMB.

#### *AMB induces MIP-1α and MIP-1β expression in hPBMCs*

In an effort to verify data obtained by cDNA microarray analysis, we measured the concentrations of three representative gene products in cell supernatants in response to AMB. Only three of the initial five donors were available for

hPBMC isolation in these studies. hPBMCs were exposed to AMB 5 µg/mL or medium alone for 6 h, at which point IL-1β, MIP-1α and MIP-1β concentrations were measured by ELISA. IL-1β concentrations increased in hPBMCs from two of the three donors, whereas MIP-1α and MIP-1β concentrations increased in cells from all donors in response to AMB (Table 3). These supernatant concentrations correlated with mRNA levels.

## Discussion

In the present study, it has been demonstrated that AMB induces the expression of several genes encoding immunomodulatory proteins in THP-1 cells and hPBMCs. Furthermore, there are both similarities and differences between the responses observed with these two types of cell, as well as variability between donors of hPBMCs. AMB induced the expression of IL-1α and the chemokines IL-8, MIP-1α and MIP-1β in both THP-1 cells and hPBMCs. Other immunomodulatory genes newly found to be up-regulated in response to AMB in THP-1 cells in the present study were those encoding the signal transduction proteins nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor α (NFKBIA) and v-jun (AP-1) and the TNF-α-inducible proteins 3 and 6. Genes that were AMB responsive in hPBMCs that have been shown to be responsive in THP-1 cells in previous studies include those encoding the cytokines IL-1β and its soluble receptor antagonist IL-1Ra. Other immunomodulatory genes

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**Table 3.** Cell supernatant concentrations (pg/mL) of MIP-1 $\beta$ , MIP-1 $\alpha$  and IL-1 $\beta$  from hPBMCs isolated from subjects 2, 4 and 5 exposed to AMB 5  $\mu$ g/mL for 6 h

	Subject 2			Subject 4			Subject 5		
	MIP-1 $\beta$	MIP-1 $\alpha$	IL-1 $\beta$	MIP-1 $\beta$	MIP-1 $\alpha$	IL-1 $\beta$	MIP-1 $\beta$	MIP-1 $\alpha$	IL-1 $\beta$
Medium	1117.5	616.5	32.89	828	722.5	31.48	737	776	31.13
5 $\mu$ g/mL AMB	13392	7964.5	371.49	2405	2816.5	45.16	2327.5	4071.5	102.35

uniquely found to be up-regulated in response to AMB in hPBMCs include those encoding COX-2, G6PD and TRAF1.

IL-8 is a CXC chemokine capable of chemoattracting and activating neutrophils.<sup>17</sup> MIP-1 $\alpha$  and MIP-1 $\beta$  are closely related CC chemokines that are implicated in a number of immune and inflammatory responses.<sup>18</sup> Both attract monocytes and attract and induce the adhesion of T lymphocytes. MIP-1 $\alpha$ , but not MIP-1 $\beta$ , is chemoattractant for B lymphocytes, eosinophils and basophils.<sup>19,20</sup> MIP-1 $\beta$  also has the capacity to degranulate eosinophils and induce histamine release from basophils and mast cells. The genes encoding IL-8 and MIP-1 $\beta$  were previously shown to be AMB-responsive in THP-1 cells.<sup>13</sup> However, this is the first study to demonstrate AMB-induced MIP-1 $\alpha$  mRNA or protein production.

Production of these chemokines in response to AMB may explain the chemotactic effects observed in monocytes, macrophages and neutrophils. The ability of IL-8 to recruit neutrophils could mediate the pulmonary toxicity occasionally observed during administration of AMB. The lipid formulations of AMB deliver larger amounts of drug to the pulmonary tissue.<sup>21</sup> It is conceivable that enhanced pulmonary neutrophil recruitment in response to elevated local concentrations of IL-8 could lead to pulmonary leukostasis consistent with the pulmonary toxicity associated with AMB preparations. Indeed, studies in animal models have demonstrated that AMB pulmonary toxicity involves neutrophil recruitment to the lungs.<sup>22,23</sup> MIP-1 $\beta$ -induced release of histamine from basophils and mast cells could also be involved in the infusion-related toxicity observed in some individuals and may explain the anecdotal efficacy of the antihistamine, diphenhydramine, as prophylaxis for this toxicity.

The variability of these adverse reactions may correlate with the variability of production of these chemokines among individuals, as has been observed with infusion-related toxicity and IL-1 $\beta$  and TNF- $\alpha$  production.<sup>10</sup> Although no definite conclusions can be drawn from the limited number of donors studied here, it is noteworthy that changes in gene expression in response to AMB were variable between donors. In the present study, mRNA levels of both MIP-1 $\beta$  and IL-8 in response to AMB were particularly high in the hPBMCs of two donors compared with the other three, whereas mRNA levels for MIP-1 $\alpha$  remained similar among all five donors.

IL-1 $\beta$  mRNA levels were elevated in hPBMCs from three donors compared with the other two. Of the three donors for whom cytokine responses were assessed, one exhibited only minimal IL-1 $\beta$  production in response to AMB (Table 3), whereas cells from all three subjects produced MIP-1 $\alpha$  and MIP-1 $\beta$ . This suggests that the effect of AMB on these two chemokines is independent of IL-1 $\beta$  production, although a role for IL-1 $\alpha$  cannot be excluded.

IL-1 $\alpha$  and IL-1 $\beta$  are structurally similar products of two separate genes.<sup>24</sup> A portion of the IL-1 $\alpha$  precursor is retained intracellularly whereas some is transported to the cell surface and becomes associated with the cell membrane.<sup>25</sup> Both forms of IL-1 bind type I and type II receptors found on a large variety of cells. IL-1 elicits a multitude of effects, including fever. In endothelial cells and smooth muscle, IL-1 induces production of prostaglandin synthesis.<sup>26</sup> In the liver, IL-1 increases hepatic protein synthesis and activates synthesis of acute phase proteins.<sup>27</sup> IL-1 induces fever, slow wave sleep and release of corticotropin-releasing factor and adrenocorticotropin.<sup>25,26,28</sup> It also elicits a myriad of immune effects in monocytes/macrophages, T and B lymphocytes and NK cells.<sup>24</sup> AMB-induced IL-1 $\beta$  mRNA and protein production have been demonstrated in THP-1 cells. The expression ratios for the gene encoding IL-1 $\beta$  in the present study using THP-1 cells were just below the predetermined threshold (1.96). This is not surprising, as peak IL-1 $\beta$  mRNA levels are observed at ~4 h after exposure to AMB in these cells.<sup>12</sup> Along with IL-1 $\beta$ , IL-1 $\alpha$  may mediate the infusion-related fever and chills associated with AMB.

IL-1Ra is a soluble receptor antagonist produced by a multitude of cells, including monocytes and macrophages.<sup>24</sup> Its release can be induced by IL-1 and is believed to attenuate the deleterious effects of this cytokine family. In THP-1 cells, IL-1Ra has been observed to respond to AMB after 24 h.<sup>12</sup> This study demonstrates that the IL-1Ra response occurs as early as 2 h in hPBMCs. As would be expected, IL-1Ra mRNA expression was highest in hPBMCs with increased IL-1 $\beta$  mRNA expression. AMB-induced IL-1Ra may account for the attenuation of AMB-induced infusion-related toxicity observed on administration of subsequent doses.

Whereas TNF- $\alpha$  is not represented on the arrays used in this study, AMB did induce expression of the genes encoding TNF- $\alpha$ -inducible proteins (TNFAIPs) 3 and 6. TNFAIP6 is a

member of the hyaladherin family of proteins and is believed to be involved in cell–cell and cell–matrix interactions during inflammation, whereas TNFAIP3 elicits a number of effects discussed below.<sup>29</sup>

Transcription factors known to be involved in the induction of proinflammatory cytokine response include nuclear factor  $\kappa$ B (NF- $\kappa$ B) and AP-1. Several genes associated with these transcription factors were found to be up-regulated in response to AMB. These included the NF- $\kappa$ B-induced genes I $\kappa$ B- $\alpha$  and TNFAIP3, the gene products of which act as inhibitors of this transcription factor.<sup>30</sup> I $\kappa$ B- $\alpha$  binds NF- $\kappa$ B in its inactive cytoplasmic form, where, on induction, phosphorylation of I $\kappa$ B- $\alpha$  occurs. This results in degradation and dissociation of I $\kappa$ B- $\alpha$  from NF- $\kappa$ B, allowing it to be translocated to the nucleus, where it initiates transcription of specific genes.<sup>31</sup> In addition to inducing mediators of inflammation, NF- $\kappa$ B also has an anti-apoptotic function.<sup>30</sup> TNFAIP3 has been shown to inhibit apoptosis in a number of cell types and has been associated with inhibition of phospholipase A2, C and D activity. Up-regulation of these genes suggests activation of NF- $\kappa$ B in response to AMB and may represent negative feedback of this process.

AP-1 is a dimeric transcription factor composed of Jun, Fos or activating transcription factor and is involved in activating transcription of a number of proinflammatory and apoptotic genes.<sup>32</sup> Up-regulation of Jun implicates this transcription factor in regulation of the monocyte response to AMB.

COX-2 is one of two isoforms of the key enzyme regulating prostaglandin production from arachidonic acid.<sup>33</sup> Whereas COX-1 is constitutively expressed, COX-2 is inducible by a number of stimuli.<sup>34</sup> AMB has been shown to cause the release of PGE<sub>2</sub> in human and murine macrophages. Such PGE<sub>2</sub> release is believed to mediate the fever and chills observed on administration of AMB by acting at the pre-optic anterior hypothalamus and altering the hypothalamic set-point. It has been suggested that PGE<sub>2</sub> release is likely in response to AMB-induced IL-1 $\beta$  in these cells. However, in the present study, COX-2 was up-regulated in response to AMB in hPBMCs from essentially all five subjects, whereas IL-1 $\beta$  mRNA and IL-1 $\beta$  protein were not. This suggests that COX-2 induction in response to AMB may actually be independent of IL-1 $\beta$ .

The gene encoding G6PD was found to be up-regulated in hPBMCs in response to AMB. This is the rate-limiting enzyme of the hexose monophosphate shunt, which is key in the production of NADPH and five-carbon sugars.<sup>35</sup> Increased G6PD has also been observed in proliferating cells, after oxidative stress and in response to LPS.<sup>36</sup> NADPH is necessary for the respiratory burst in neutrophils.<sup>37</sup> It is therefore possible that increased G6PD in response to AMB may in part explain the favourable effects it has on these effector cells.

In summary, we have demonstrated that AMB induces the expression of the genes encoding MIP-1 $\alpha$  and MIP- $\beta$ , as well

as their respective gene products, in human mononuclear cells. We have also shown that AMB induces the expression of the gene encoding IL-1 $\alpha$  in both THP-1 cells and hPBMCs, and we have found several genes with expression in response to AMB that is unique to either one of these cell types. Furthermore, we have shown that variability exists in hPBMCs in the expression of many of these genes in response to AMB. A number of these genes represent potential mediators of the immunomodulatory properties and infusion-related toxicity associated with AMB. Correlation of the variability in expression of these genes with that observed with AMB-associated adverse events, or with therapeutic responses, would enhance our understanding of the pharmacological and toxicological properties of AMB. Further evaluation of these genes and their respective gene products in the context of the activity and toxicity of AMB is warranted.

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