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# RESEARCH PAPER

# The impact of cytokines on the expression of drug transporters, cytochrome P450 enzymes and chemokine receptors in human PBMC

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**Background and purpose:** The function of transporters in peripheral blood mononuclear cells (PBMC) has been characterized, but less is known about cytochrome P450 (CYP) enzyme function in these cells. Given that cytokines are dysregulated in many diseases, the purpose of this work was to assess the impact of cytokines on the expression of CYPs, transporters and chemokine receptors in PBMC.
**Experimental approach:** Human PBMC were incubated with cytokines for 48 h. ATP-binding cassette (ABC)B1, ABCC1, ABCC2, CYP2B6, CYP3A4, CXCR4 and CCR5 expression were measured by quantitative polymerase chain reaction and flow cytometry at 0, 4, 8, 24 and 48 h. Enzyme activity was assessed using fluorescent probes.
**Key results:** We show here functional activity of CYP3A4 and CYP2B6 in PBMC. Furthermore, cytokines had a significant impact on the mRNA and protein expression of all proteins. For example, interleukin-2 (IL-2) had a marked impact on ABCB1 mRNA (% control 4745 ± 11961) and protein (% control 200 ± 57). Increases in drug efflux transporter expression, in response to cytokines, resulted in reduced cellular accumulation of digoxin [decrease of 17% and 26% for IL-2 and interferon-γ (IFNγ) respectively] and saquinavir (decrease of 28% and 30% for IL-2 and IFNγ respectively). The degree to which drug transporter and chemokine receptor expression changed in response to cytokines was positively correlated (e.g. ABCB1 and CXCR4, $r^2 = 0.545$).
**Conclusions and implications:** These data have important implications for diseases in which cytokines are dysregulated and for which pharmacological intervention targets immune cells.
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**Keywords:** ABCB1 (P-glycoprotein); ABCC1 (MRP1); ABCC2 (MRP2); CYP2B6; CYP3A4; CXCR4; CCR5; cytokines; peripheral blood mononuclear cell; multidrug resistance

**Abbreviations:** ABC, ATP-binding cassette; CYP, cytochrome P450; PBMC, peripheral blood mononuclear cells; SNP, single nucleotide polymorphism

## Introduction

Drug metabolism is an important aspect of drug disposition. Cytochrome P450 (CYP) enzymes are probably the most important effectors of oxidative metabolism in humans. Most CYPs can metabolize multiple substrates, and many can catalyse multiple reactions. This accounts for their central importance in metabolizing an extremely large number of endogenous and exogenous molecules. In the liver, these substrates include xenobiotics, but also endobiotics such as bilirubin. CYP enzymes are present in many other tissues of the body including the mucosa of the gastrointestinal tract (McFadyen *et al.*, 2004). Few studies have investigated CYP isoforms in lymphocytes, potentially important as a number of drug therapies target the lymphocyte e.g. highly active antiretroviral therapy. However, previous studies have indicated that CYP2B6 and CYP3A4 mRNA are expressed in lymphocytes (Hukkanen *et al.*, 1997; Haas *et al.*, 2005). Therefore, we first assessed whether functional CYPs, capable of metabolism, are expressed in this important cell type. In addition, we performed a proof of concept study to assess the impact of genetic polymorphisms on mRNA expression in these cells.

ATP-binding cassette (ABC) transporters represent a large family of transmembrane proteins that are also important determinants of drug disposition. The energy provided by

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ATP hydrolysis is used to drive the transport of various compounds across the cell membrane. ABC drug transporters in the intestine, liver and kidney have a large impact on the pharmacokinetics of numerous drugs. In addition, ABC transporters can actively restrict drug distribution to the site of action (e.g. lymphocytes) and thereby modulate the effectiveness of drug therapy (Owen and Khoo, 2004; Janneh *et al.*, 2005; 2007; Detsika *et al.*, 2007). The activity of ABC transporters is highly variable and can be affected within hours by inhibitors and days by inducing agents (Albermann *et al.*, 2005). Also, genetic influences (Owen and Khoo, 2004) such as single nucleotide polymorphisms (SNPs) and endogenous factors such as hormones have been shown to affect ABC transporter expression and activity.

Probably the best-studied ABC transporter is ABCB1 (P-glycoprotein). ABCB1 is capable of expelling many hydrophobic compounds including immunosuppressive agents (e.g. cyclosporin A), statins (Holtzman *et al.*, 2006), methotrexate and glucocorticoids (Richaud-Patin *et al.*, 2004). Similar to ABCB1, ABCC1 has been shown to have wide substrate specificity. ABCC1 is present in most tissues including hepatic and intestinal tissues and peripheral blood cell types (Zaman *et al.*, 1993; Burger *et al.*, 1994). Transporters have also been implicated in failure to treat autoimmune diseases such as myasthenia gravis (MG) and rheumatoid arthritis (RA), where incomplete penetration of the drug is responsible for unsuccessful therapy. In the latter case it has been shown that patients with refractory RA are those with the highest lymphocyte ABCB1 functional activity, and this correlates with tumour necrosis factor-α (TNFα) mRNA (Tsujimura *et al.*, 2008). Furthermore, we recently showed that ABCC1 expression is down-regulated in lymphocytes in RA patients treated with methotrexate (Hider *et al.*, 2006).

Despite their role in transporting drugs, the normal physiological role of ABC transporters in lymphocytes still requires clarification. Possible endogenous, cellular functions include regulation of intracellular pH and possible chloride channel function (de Lange, 2004). More recent evidence suggests ABCB1 may confer resistance to apoptosis by interfering with caspase-8 activation (Ruefli *et al.*, 2002). A role in the secretion of cytokines (Vasquez *et al.*, 2005) has also been suggested which may account for its expression on these cells (Drach *et al.*, 1996).

We previously reported a positive correlation between the expression of ABCB1 and the chemokine receptor CXCR4 in peripheral blood mononuclear cells (PBMC) from both healthy volunteers (Owen *et al.*, 2004b) and HIV+ patients (Chandler *et al.*, 2007). CXCR4 is involved in the recruitment and subsequent chemotaxis of immune cells to sites of inflammation. CXCR4 is a coreceptor for the more cytopathic T-tropic strain of HIV (Berkowitz *et al.*, 1998) and has also been implicated in the metastasis of malignant cells (Kucia *et al.*, 2005). In addition, a strong correlation between expression of ABCC1 and CXCR4 protein was observed (Chandler *et al.*, 2007). Differences in the pharmacokinetics of many antiretrovirals between HIV-infected persons and healthy volunteer controls have been reported (Dickinson *et al.*, 2008). This has been attributed to a number of factors observed in HIV patients such as higher gastric pH (Welage *et al.*, 1995), higher α1-acid glycoprotein concentrations (Merry *et al.*, 1996) and a greater degree of variability in CYP3A activity (Slain *et al.*, 2000; Fellay *et al.*, 2005). However, differences in cytokine profiles have also been demonstrated between healthy volunteers and HIV+ patients (Becker, 2004a).

Cytokines are important chemical messengers *in vivo* that act through receptors and secondary messengers. Particularly important in the immune response, they participate in governing how the body deals with non-self molecules. Studies on both $T_h1$ [e.g. interleukin-2 (IL-2), IL-12 and interferon-γ (IFNγ)] and $T_h2$ (e.g. IL-4, IL-10 and IL-13) cytokine production have revealed an aberration in HIV-1 infected individuals exemplified by increased expression of cytokines and a gradual bias associated with a $T_h2$ cytokine profile over the course of chronic infection (Becker, 2004b). Cytokine dysregulation has also been implicated in rheumatoid arthritis (Canete *et al.*, 2000) and cancer (Howell and Rose-Zerilli, 2007).

Given that transporters and chemokine receptors appear co-ordinately regulated and that cytokines play a pivotal role in the immune response in a number of diseases, it was of interest to gain a coherent understanding of the impact that cytokines have on the expression of these proteins in lymphocytes. The aims of this study were therefore to investigate the effects of cytokines on the expression of transporters, CYPs and chemokine receptors, over a period of 48 h. This was investigated at both the mRNA and protein level using real-time quantitative polymerase chain reaction (QPCR) and flow cytometry respectively.

## Methods

All drug/molecular target nomenclature conforms with BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

*Isolation and preparation of PBMC*

Venous blood samples (60 mL) were obtained from healthy volunteers via venopuncture. Samples were layered onto Lymphoprep and separated by density gradient centrifugation according to the manufacturer's instructions. Cell count was determined using a Chemometec nucleocounter. For confirmation of CYP2B6 and CYP3A4 expression and activity, PBMC were obtained from four healthy volunteers. For analysis of the impact of CYP polymorphisms on mRNA expression of CYP2B6 and CYP3A4, genomic DNA and cDNA were obtained from PBMC of 20 healthy volunteers. For the impact of cytokines, PBMC from six volunteers were used per incubation.

*CYP2B6 and CYP3A4 activity in PBMC*

Fluorescence microscopy was utilized in order to assess whether CYP2B6 and CYP3A4 were functional within PBMC. This was conducted in the presence or absence of the CYP3A4 inhibitor, ritonavir (10 $\mu mol \cdot L^{-1}$) and the CYP2B6 inhibitor, orphenadrine (10 $\mu mol \cdot L^{-1}$) (Guo *et al.*, 1997; Soars *et al.*, 2006). Cells were adhered to coverslips, previously coated in poly – L-lysine and loaded onto a Bioptechs perfusion chamber maintained at 37°C. At time zero, cells were then perfused with Invitrogen fluorescent vivid CYP probes [either 5 $\mu mol \cdot L^{-1}$

dibenzyloxymethylfluorescein (DBOMF) (for CYP3A4) or 2 μmol·L$^{-1}$ BOMFC (for CYP2B6) in RPMI media containing 10% fetal calf serum (FCS)]. The cells were scanned for 10 min (at 1 min intervals) on a Zeiss LSM Pascal confocal microscope. For DBOMF, fluorescence (green pseudocolour) was excited using the 488 nm line of an argon laser and collected off an NFT 545 dichroic mirror through a 505–530 nm bandpass filter. For BOMFC, fluorescence (blue pseudocolour) was excited using a mercury vapour lamp and visible light was analysed using pseudo 4,6-diamine-2-phenylindole settings.

*CYP2B6 and CYP3A4 genotyping*

For CYP2B6 genotyping, pre-amplification for exon 4 and exon 9 was first conducted to discriminate from the *CYP2B6* pseudogene (*CYP2B7*) by modification of previously reported methods (Lang *et al*., 2001). Genotyping for CYP2B6 516G > T (rs3745274) and 1459C > T (rs3211371) was performed on the resultant amplicons and genotyping for CYP3A4 (–392A > G; rs2740574) and CYP3A5 (6893A > G; rs776746) was conducted on genomic DNA by real-time PCR allelic discrimination using standard methodology (95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) in a DNA Engine Opticon 2 system. Primer and probe sequences are available on request.

*Treatment of PBMC with cytokines*

Peripheral blood mononuclear cells were resuspended in media (RPMI-1640 containing 15% FCS, $2 \times 10^6$ cells·mL$^{-1}$) and incubated with relevant cytokines at a concentration of 10 ng·mL$^{-1}$ in a humidified incubator (37°C, 5% $CO_2$) consistent with previous studies (Ottonello *et al*., 2002; Belliard *et al*., 2004; Odamaki *et al*., 2004). Cytokines included were: IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IFNγ, transforming growth factor-β and TNFα. Control incubations consisted of untreated PBMC in media at each time point to enable relative comparison. After a period of 0, 4, 8, 24 and 48 h, cells ($2 \times 10^6$) were isolated, resuspended in Hanks balanced salt solution (HBSS) and centrifuged (300× *g*, 6 min). The resultant cell pellet was resuspended in Tri reagent (1 mL) for subsequent RNA extraction or CellFIX for protein analysis via flow cytometry.

*Analysis of transporter and coreceptor expression by real-time QPCR*

Following reverse transcription, samples were prepared for real-time QPCR; 40 ng cDNA was combined with universal master mix, sense and antisense primers (0.4 μmol·L$^{-1}$ each) and oligonucleotide probe (0.2 μmol·L$^{-1}$). Assays on demand primer and probe mixes, for ABCC1 (Hs00219905_m1), ABCC2 (Hs00166123_m1) and CCR5 (Hs00152917_m1), were received ready mixed from Applied Biosystems. The Applied Biosystems Assays by design service was used to provide primers and probes for GAPDH (Forward primer 5′-GAAGG TGAAGGTCGGAGTC-3′,Reverse primer 5′-GAAGATGGTG ATGGGATTTC-3′, Probe sequence 5′-CAAGCTTCCCGTTCTC AGCC-3′), ABCB1 (Forward primer 5′-GGAAGCCAATGCCTA TGACTTTAT-3′,Reverse primer 5′-TCAACTGGGCCCCTCT CTCT-3′, Probe sequence 5′-TGAAACTGCCTCATAAATTTGA CACCCTGG-3′) and CXCR4 (Forward primer, 5′-CACCGCA TCTGGAGAACCA-3′, Reverse primer, 5′-CCCATTTCCTCG GTGTAGTTATCT-3′, Probe sequence, 5′-CACCGCATCTGG AGAACCA-3′). GAPDH was included as a housekeeping gene. Thermal cycling conditions for all assays consisted of 15 min at 95°C followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. Quantification of PCR products occurred in real time and was analysed using a Bio-Rad Chromo4 real-time QPCR machine. Expression data were normalized to GAPDH expression using the comparative δδCt method (Owen *et al*., 2004a,b).

*Optimization of flow cytometry assays for ABCC2, CYP2B6 and CYP3A4*

Flow cytometry assays were optimized for ABCC2, CYP2B6 and CYP3A4 as previously described (Liptrott *et al*., 2008). Samples were analysed on a Coulter Epics XL-MCL flow cytometer. Forward scatter and side scatter were detected on a linear scale to aid gating of viable cells and fluorescence on a logarithmic scale. Median fluorescence of the isotype control (IC) stained cells was subtracted from that of test antibody stained cells to yield relative fluorescence units (RFU) for relative expression.

*Analysis of transporter, CYP and chemokine receptor expression in PBMC by flow cytometry*

Following incubation PBMC were washed in PBS (1×) and cell density corrected to $2 \times 10^6$ cells·mL$^{-1}$; 100 μL of cell suspension was transferred into corresponding wells of a 96 well plate. The plate was then centrifuged (300× *g*, 10 min) and the supernatant carefully removed. Cells were then resuspended in CellFIX (100 μL) for 30 min at 4°C. Cells were subsequently washed twice in PBS and incubated with monoclonal antibodies against; ABCB1, ABCC1, ABCC2, CCR5 or CXCR4. Cells were also incubated separately with matched IC antibodies to control for non-specific binding. Following incubation with primary antibody, or IC, cells were washed (3×) in PBS and incubated with an appropriate secondary antibody for 1 h. After a final three washes cells were transferred to appropriate tubes and analysed using a Coulter Epics XL-MCL flow cytometer. Forward scatter and side scatter were detected on a linear scale to aid gating of viable cells and fluorescence on a logarithmic scale. Median fluorescence of the IC stained cells was subtracted from that of test antibody stained cells to yield RFU.

*Evaluation of the impact of increased transporter expression on the intracellular accumulation of digoxin and saquinavir in PBMC treated with IL-2 and IFNγ*

Peripheral blood mononuclear cells were isolated from whole blood of healthy volunteers ($n = 3$) via density gradient centrifugation and resuspended to $5 \times 10^6$ cells·mL$^{-1}$. PBMC were incubated in media containing IL-2 or IFNγ at a concentration of 10 ng·mL$^{-1}$ for 24 h. In order to assess the intracellular accumulation of digoxin and saquinavir PBMC were resuspended in media containing [$^3$H]-digoxin (0.025 μCi·mL$^{-1}$) or [$^3$H]-saquinavir (0.33 μCi·mL$^{-1}$). Cells were incubated for

20 min, centrifuged (13 500× *g*, 1 min) and 100 μL aliquots of supernatant taken into scintillation vials. The remaining supernatant was discarded, the pellet was washed three times (HBSS, 13 500× *g*, 1°C, 1 min) and then dissolved in water (100 μL). Scintillation fluid (4 mL) was then added to both supernatant and pellet and the cellular accumulation was calculated (ratio of radiolabelled drug associated with the cell pellets divided by that associated with media). A cell volume of 0.4 pL was assumed. The accumulation of digoxin and saquinavir was determined at 0 h and 24 h post incubation with cytokines. Controls consisted of PBMC incubated in media in the absence of cytokines.

*Materials*

RPMI 1640, chloroform, Tri reagent and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Poole, UK). Isopropyl alcohol and ethanol were obtained from Fisher Scientific (Loughborough, UK). All cytokines were purchased from Insight Biotechnology (Middlesex, UK). QPCR Master Mix was purchased from ABgene (Epsom, UK). Taqman reverse transcrption reagents, primers and probes for real-time QPCR were purchased from Applied Biosystems (Warrington, UK). CellFix was purchased from BD Bioscience (Oxford, UK). Antibodies used for flow cytometric analysis were as follows; P-glycoprotein primary antibody (UIC2 clone, Immunotech, Marseilles, France), ABCC1 primary antibody (QCRL-1 clone, Calbiochem, San Diego, USA), ABCC2 primary antibody (M2III-6 clone, AbCam, Cambridge, UK), CCR5 primary antibody (rat anti-human CD195, Serotec, Oxford, UK) and CXCR4 primary antibody obtained from NIBSC AIDS reagent project (Herfordshire, UK). Radiolabelled saquinavir ($^3$H, 99.9% estimated purity) was purchased from Moravek Chemicals, USA. Radiolabelled digoxin ($^3$H, 99.8% estimated purity) was purchased from American radiolabelled chemicals Inc. (Missouri, USA). The DNA Engine Opticon 2 system was from MJ Research Inc. (USA).

*Statistical analysis*

For the influence of CYP genotype on mRNA expression of CYPs, data are given as mean ± s.e. in each genotype group. For the effect of cytokines, data are expressed as mean percentage change compared with controls ± SD. For both, normality of the data was assessed using a Shapiro-Wilk test and statistically significant differences determined using the Mann–Whitney *U*-test for all data. A $P < 0.05$ was considered statistically significant. Correlations in the degrees of change of expression were also analysed. This was conducted using a Spearman's Rank Correlation on log transformed data with the difference between the 0 h and 4 h time points.

## Results

*CYP2B6 and CYP3A4 activity in PBMC*

In order to determine the functional activity of CYP2B6 and CYP3A4 PBMC were incubated with fluorescent substrates for CYP2B6 and CYP3A4. PBMC were able to metabolize the DBOMF (CYP3A4; Figure 1A) and BOMFC (CYP2B6; Figure 1C) vivid substrates and this metabolism was inhibited by the addition of ritonavir (Figure 1B) and orphenadrine (Figure 1D) respectively.

*Impact of CYP genotype on CYP mRNA expression in PBMC*

The allele frequencies for CYP2B6 516T, CYP2B6 1459T, CYP3A4*1B and CYP3A5*3 polymorphisms were 17.5%, 12.5%, 0% and 7.5% respectively (Table 1). For mRNA the median (range) expression was 2.8 (2.3–22.9) for CYP2B6 and 2.3 (2.3–11.7) for CYP3A4 and no correlation was observed between CYP2B6 and CYP3A4 mRNA ($r^2 = 0.01$; $P = 0.67$). A significant association was observed between 516G > T and lymphocyte CYP2B6 expression (10.1 ± 2.4 in G homozygotes versus 2.3 ± 0.03 in heterozygotes; $P = 0.003$; 95% CI = 3.4, 12.2) which remained significant after correction for multiple comparisons ($P = 0.012$). No differences in CYP2B6 or CYP3A4 mRNA expression were observed for the other SNPs (Table 1).

*The effects of $T_h1$ cytokines on expression of ABCB1, ABCC1, ABCC2, CXCR4 and CCR5*

$T_h1$ cytokines had a number of statistically significant effects on transporters, CYP enzymes and chemokine receptors. Figure 2 shows the impact of IL-2, IL-12 and IFNγ on the expression of mRNA and protein. ABCB1, ABCC1, ABCC2, CXCR4 and CCR5 mRNA expression all increased in response to these cytokines. However, CYP2B6 and CYP3A4 expression decreased. The most marked effects were seen after 4 h incubation.

Similar effects were also seen at the protein level. CYP2B6 and CYP3A4 protein levels decreased after the 8 h incubation with $T_h1$ cytokines. Expression of ABCB1, ABCC1, ABCC2, CXCR4 protein increased after 24 h incubation with cytokines.

*The effects of $T_h2$ cytokines on expression of ABCB1, ABCC1, ABCC2, CXCR4 and CCR5*

Figure 3 shows the impact of $T_h2$ cytokines on the expression of transporters, CYP enzymes and chemokine receptors. $T_h2$ cytokines increased expression of ABCB1, ABCC1, ABCC2, CXCR4 and CCR5 mRNA. The $T_h2$ cytokines IL-4 and IL-13 were the only cytokines to affect mRNA expression of CYP2B6 and CYP3A4.

CYP2B6 and CYP3A4 protein levels were not affected until 24 h after incubation with cytokines. $T_h2$ cytokines generally increased the protein expression of CYP2B6 and CYP3A4. The most significant increase was seen with IL-4 24 h *post* incubation.

*Non-$T_h1$/$T_h2$ cytokine effects on the expression of ABCB1, ABCC1, ABCC2, CXCR4 and CCR5*

The cytokines designated in this study as non-$T_h1$/$T_h2$ had the least impact on the expression of transporters, CYP enzymes and chemokine receptors, as can be seen in Figure 4. The greatest impact appeared to be on ABCC1 and ABCC2 at both the mRNA and protein level. CXCR4 and CCR5 mRNA levels

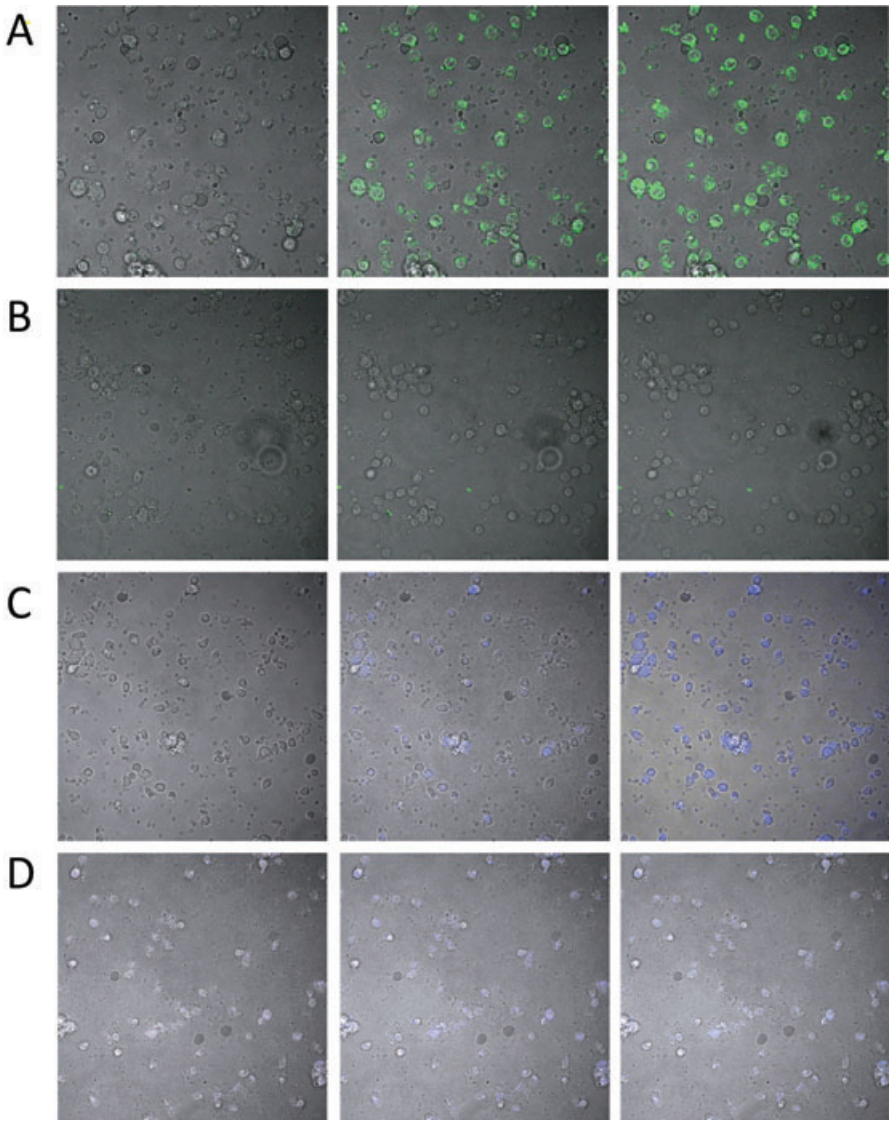


**Figure 1** Metabolism of fluorescent CYP substrates in PBMC. Representative PBMC turnover of vivid DBOMF substrate (CYP3A4) in the absence (A) and presence (B) of ritonavir (10 μmol·L$^{-1}$) at 0, 2 and 4 min respectively. Representative PBMC turnover of vivid BOMFC substrate (CYP2B6) in the absence (C) and presence (D) of orphenadrine (10 μmol·L$^{-1}$) at 0, 5 and 10 min respectively. CYP, cytochrome P450; DBOMF, dibenzyloxymethylfluorescein; PBMC, peripheral blood mononuclear cells.

were affected by these cytokines but the effects were not as marked at the protein level. CYP2B6 mRNA and protein levels were increased in response to these cytokines with the most significant effects seen with TNFα. CYP3A4 mRNA and protein were only affected by IL-7.

*Relationship between the degrees of change in expression of mRNA in response to cytokines*

Overall, the most marked or statistically significant effects on mRNA levels were observed after 4 h incubations. When the degree of change in expression between the 0 and 4 h time-points was regressed between transcripts, a number of significant relationships emerged. Of particular interest, the change in expression of ABCB1 and CXCR4 in response to cytokines was positively correlated ($r^2 = 0.545$, $P = 0.0061$). This was also true of ABCC1 and CCR5 ($r^2 = 0.425$, $P = 0.0217$), ABCC2 and CCR5 ($r^2 = 0.740$, $P = 0.0003$) and ABCC1 and ABCC2 ($r^2 = 0.795$, $P < 0.0001$).

*Impact of cytokine incubation on the intracellular accumulation of digoxin and saquinavir in PBMC*

Figure 5 shows the cellular accumulation of digoxin (a) and saquinavir (b) in PBMC following incubation with IL-2 and IFNγ. In accordance with an increase in expression of efflux drug transporters, the intracellular accumulation of digoxin and saquinavir were significantly decreased. After 24 h, digoxin cellular accumulation was significantly lower than the control incubation by 17% when incubated with IL-2 and 26% lower when incubated with IFNγ. The intracellular accumulation of saquinavir was also significantly affected

**Table 1** Allele frequencies and impact of polymorphisms on expression of CYP2B6 and CYP3A4 mRNA. PBMC and genomic DNA were obtained from blood samples from 20 healthy volunteers

| *Gene* | *SNP* | *Minor allele frequency (%)* | *Log CYP2B6 mRNA* | | | *Log CYP3A4 mRNA* | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Wild-type homozygote* | *Carrier of mutant allele* | *P-value (corrected)* | *Wild-type homozygote* | *Carrier of mutant allele* | *P-value (corrected)* |
| CYP2B6 | 516G > T (rs3745274) | 17.5 | 10.1 ± 2.4 | 2.3 ± 0.03 | 0.003 (0.012) | 2.78 ± 1.07 | 2.30 ± 0.01 | 0.16 (0.64) |
| | 1459C > T (rs3211371) | 12.5 | 6.75 ± 7.69 | 8.17 ± 6.78 | 0.74 (>1.0) | 2.30 ± 0.01 | 3.62 ± 1.52 | 0.08 (0.32) |
| CYP3A4 | −392A > G (rs2740574) | 0 | 7.09 ± 7.30 | N/A | N/A | 2.61 ± 0.87 | N/A | N/A |
| CYP3A5 | 6986A > G (rs776746) | 7.5 | 7.08 ± 7.30 | 11.78 ± 8.43 | 0.44 (>1.0) | 2.61 ± 0.87 | 3.71 ± 2.44 | 0.15 (0.60) |

Data shown are means ± s.e.mean.
CYP, cytochrome P450; N/A, not available; PBMC, peripheral blood mononuclear cells; SNP, single nucleotide polymorphism.

by incubation with cytokines, IL-2 decreased the cellular accumulation of saquinavir by 28% and IFNγ decreased saquinavir cellular accumulation by 30%.

## Discussion

Many studies have illustrated that P-glycoprotein influences intracellular accumulation of many drugs (Callaghan *et al.*, 2008). Moreover, we recently reported that the efficacy of the antiretroviral drug, saquinavir, is influenced by the relative expression of P-glycoprotein (Owen *et al.*, 2004b) and that transporter inhibition lowers the emergence of drug resistant strains of HIV (Chandler *et al.*, 2007). This is important because the mechanisms that regulate the expression of CYP enzymes and ABC transporters are known to be very similar. Indeed, the pregnane X receptor (PXR) is known to act as a xenobiotic sensor, regulating expression of both P-glycoprotein and CYP3A4 (Lehmann *et al.*, 1998; Geick *et al.*, 2001). Given that we recently reported the presence of PXR in PBMC (Owen *et al.*, 2004a), it was important to establish whether functional expression of CYP enzymes were also expressed in these cells.

We show here that PBMC express functional CYP2B6 and CYP3A4. The intracellular metabolism in PBMC may be an additional factor determining the concentration of drugs at their target site. Our data also illustrate that the previously reported association between CYP2B6 516G > T and expression of the protein (Lang *et al.*, 2001) is not limited to hepatic tissue. As such, PBMC may serve as easily accessible surrogates for investigating the impact of polymorphisms on expression of CYP isoforms. Furthermore, studies are now required to relate these findings to metabolism in PBMCs and efficacy of drugs *in vivo*.

Cytokines are a critical component of the immune response. In a similar manner to hormones, they act as chemical mediators and are a method of physiological communication between cells both locally and systemically throughout the body. Cytokines are notoriously pleiotropic in nature, interact in complex networks and have a capacity for redundancy, in that loss of expression of one cytokine can be compensated for by another. The combination of these factors makes cytokines incredibly powerful chemical mediators but provides a major disadvantage when attempting to study the effects of individual cytokines on an *ex vivo* basis. Given the importance of cytokines, in many diseases, it is important to gain an understanding of how they might affect the expression of proteins that influence the outcome of drug therapies. This is particularly important in the case of lymphocytes as not only are they the 'front-line' cell of the immune response but they are also implicated in many diseases. Thus, they are the site of action of many drugs.

The presented findings are also of interest given the role of TNFα in the pathogenesis of RA (Paleolog *et al.*, 1996). TNFα, unlike other cytokines, lacks hydrophobic signal sequences and, hence, must be secreted by non-conventional mechanisms. Molecules that share this feature are transported through energy-dependent proteins (Kuchler *et al.*, 1992). Furthermore, it has been demonstrated that the

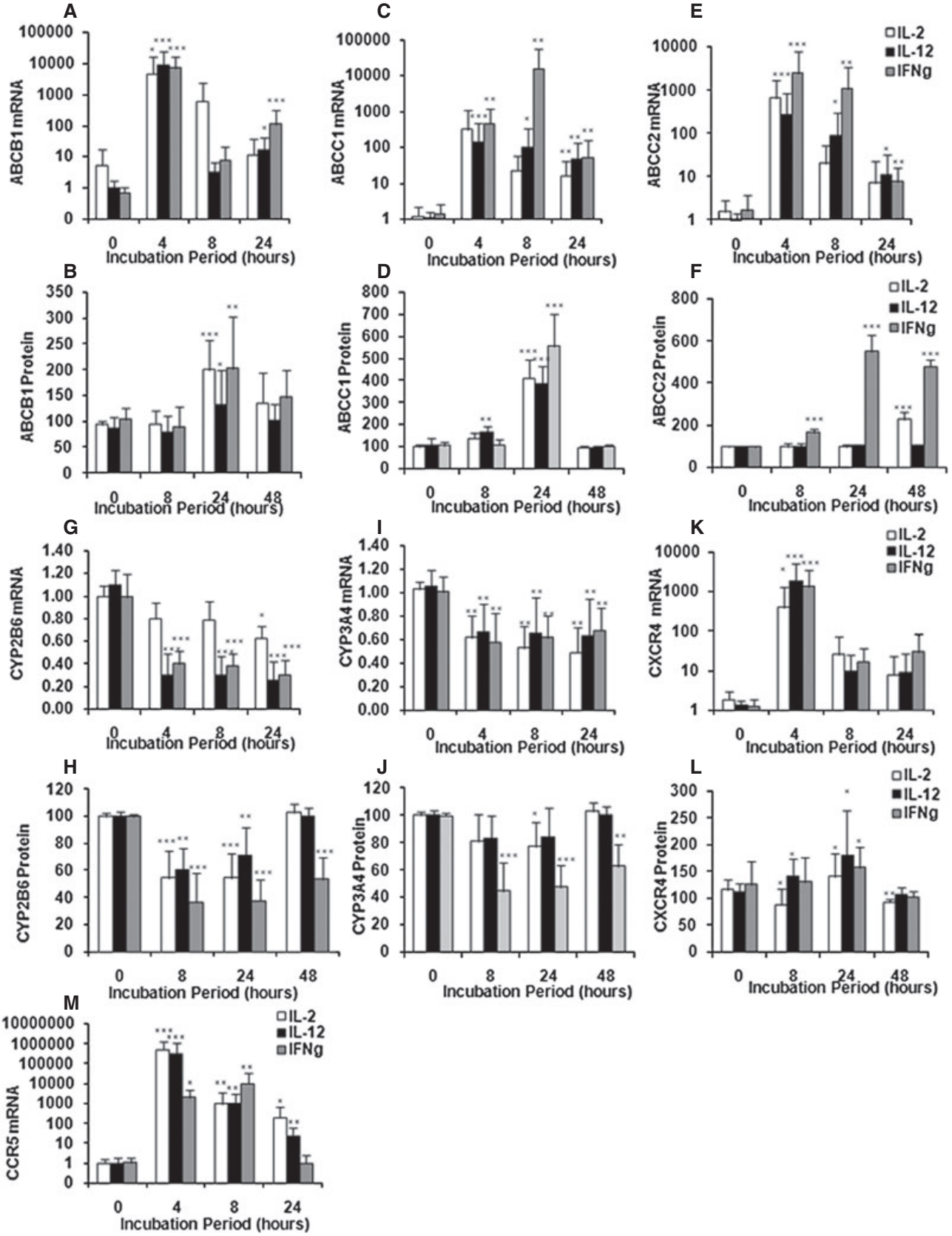


**Figure 2** Effect of $T_h1$ cytokines on the expression of transporters, CYP450s and chemokine receptors. (A), (C), (E), (G), (I), (K) and (M) The impact of $T_h1$ cytokines on ABCB1, ABCC1, ABCC2, CYP2B6, CYP3A4, CXCR4 and CCR5 mRNA levels respectively. (B), (D), (F), (H), (J) and (L) The impact of the same cytokines on protein expression of ABCB1, ABCC1, ABCC2, CYP2B6, CYP3A4 and CXCR4 respectively ($n = 8$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). ABC, ATP-binding cassette; CYP, cytochrome P450; IFN, interferon; IL, interleukin.

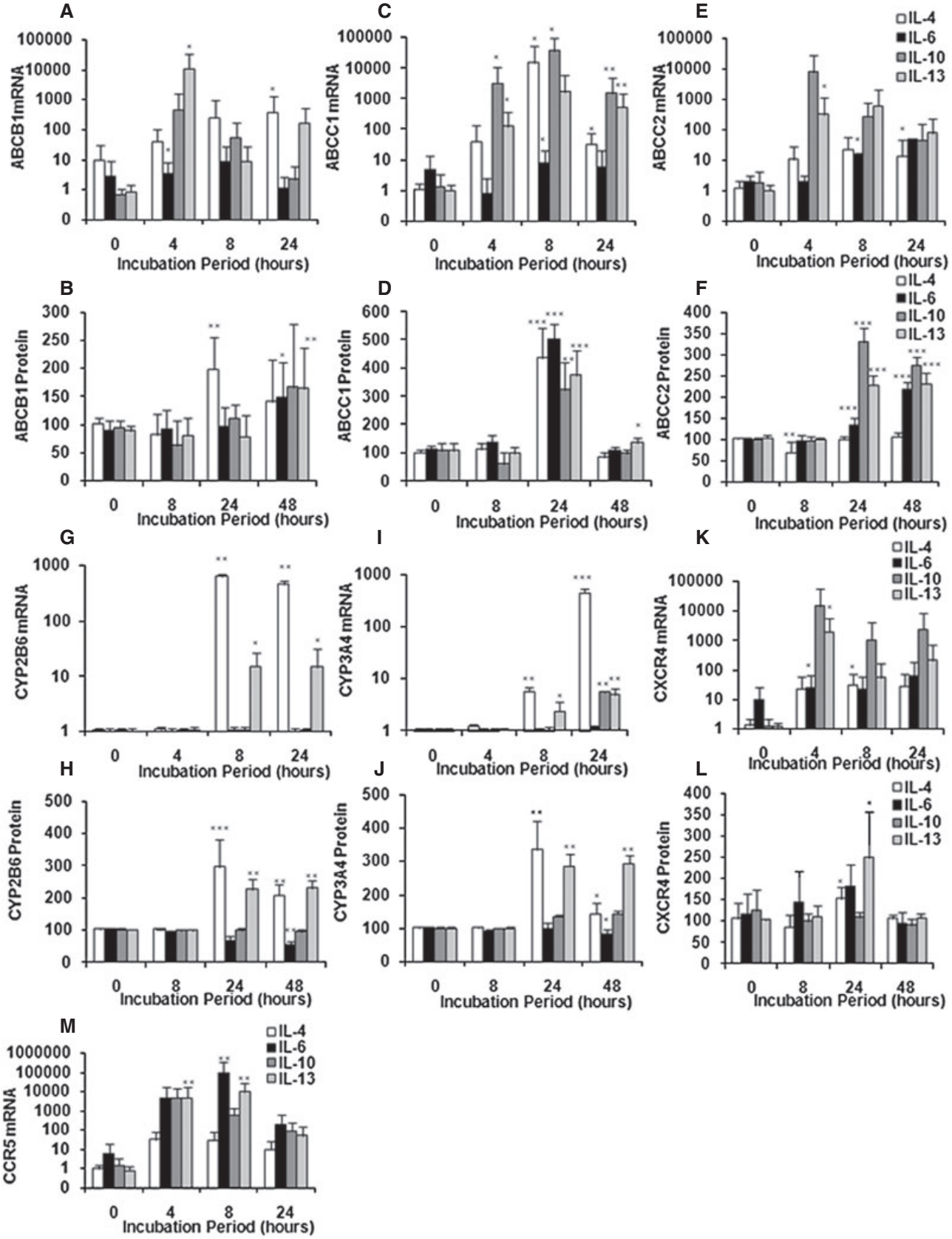


**Figure 3** Effect of $T_h2$ cytokines on the expression of transporters, CYP450s and chemokine receptors. (A), (C), (E), (G), (I), (K) and (M) The impact of $T_h2$ cytokines on ABCB1, ABCC1, ABCC2, CYP2B6, CYP3A4, CXCR4 and CCR5 mRNA levels respectively. (B), (D), (F), (H), (J) and (L) The impact of the same cytokines on protein expression of ABCB1, ABCC1, ABCC2, CYP2B6, CYP3A4 and CXCR4 respectively ($n = 8$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). ABC, ATP-binding cassette; CYP, cytochrome P450; IL, interleukin.

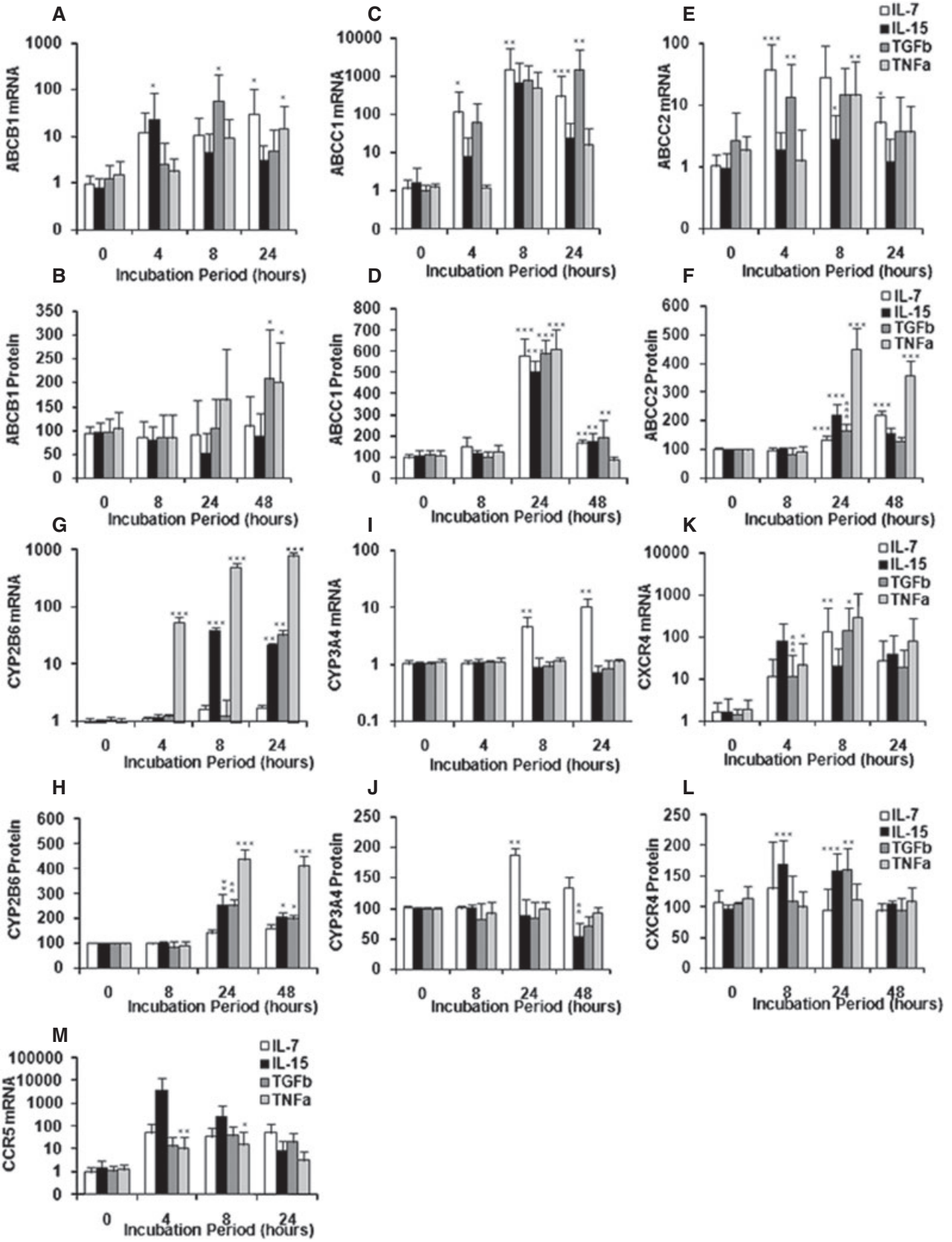


**Figure 4** Effect of non-$T_h1/T_h2$ cytokines on the expression of transporters, CYP450s and chemokine receptors. (A), (C), (E), (G), (I), (K) and (m) The impact of non-$T_h1/T_h2$ cytokines on ABCB1, ABCC1, ABCC2, CYP2B6, CYP3A4, CXCR4 and CCR5 mRNA levels respectively. (B), (D), (f), (h), (j) and (l) The impact of the same cytokines on protein expression of ABCB1, ABCC1, ABCC2, CYP2B6, CYP3A4 and CXCR4 respectively ($n = 8$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). ABC, ATP-binding cassette; CYP, cytochrome P450; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor.

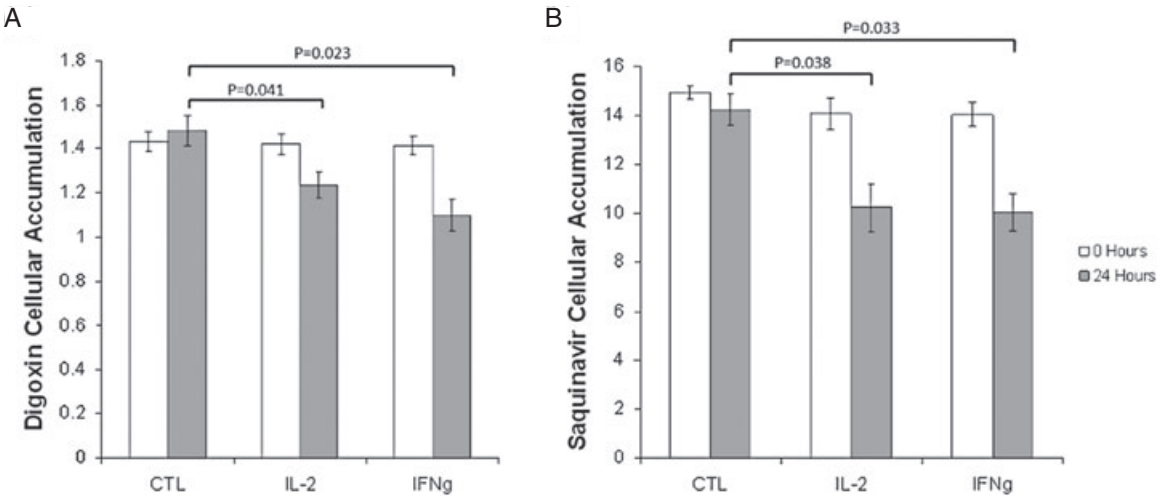


**Figure 5** Impact of cytokine incubation on the intracellular accumulation of digoxin and saquinavir in PBMC from healthy volunteers. The impact of incubation with IL-2 and IFNγ on the cellular accumulation ration of (A) digoxin and (B) saquinavir was determined at both 0 and 24 h time points ($n = 3$). CTL, control; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cells.

presence of TNFα induces *ABCB1b* gene expression and its functional activity in cultures of rat hepatocytes (Hirsch-Ernst *et al.*, 1998) and similar results were found in a human GMCSF-dependent leukaemia myeloid cell line (Bailly *et al.*, 1995). Therefore, TNFα-mediated up-regulation of ABCB1 in lymphocytes may be involved in refractory RA (Tanaka and Tsujimura, 2006).

In this study we sought to examine how cytokines affect either drug transporter, CYP or chemokine receptor expression in PBMC and gain a coherent understanding of their impact. Previous studies have reported conflicting data most likely as a result of the use of different cell types and differences in experimental design, coupled with the fact that cytokines can have different effects in different cell types (Bauer *et al.*, 2007; Blokzijl *et al.*, 2007). The cytokines used in this study were grouped into the categories $T_h1$, $T_h2$ or those not classically designated as either $T_h1$ or $T_h2$ for ease of presentation but also to examine how each profile can affect expression overall.

Both $T_h1$ and $T_h2$ cytokines had marked effects on the expression of the transporters, CYP and coreceptors included in this study. The changes in expression observed here may have functional implications with respect to the disposition of drugs used in HIV therapy and also the tropism of the virus. Cytokines involved in a $T_h2$ profile up-regulated CYP2B6 and CYP3A4, whereas $T_h1$ cytokines down-regulated their expression. This finding may be particularly important given that increased $T_h2$ profiles have been reported in later stages of HIV infection and chronological clinical studies are now required to investigate the consequences for drug metabolism. The observed relationship between changes in expression of ABCB1 and CXCR4 in response to cytokines lends support to our previous hypothesis that these genes may be co-regulated (Owen *et al.*, 2004b; Chandler *et al.*, 2007).

There are differences in the pharmacokinetics of antiretrovirals between HIV+ patients and healthy volunteers (Dickinson *et al.*, 2008). Also, there are known differences in the cytokine profiles of HIV infected persons and healthy volunteers (Becker, 2004a). Atazanavir concentrations have been shown to be significantly lower in HIV patients than healthy volunteers (Dickinson *et al.*, 2008). Incorporating the findings of the present study, the increases seen in CYP3A4 expression in response to IL-4, IL-10 and IL-13 may go some way to explaining the lower drug concentrations observed in HIV patients. However, this will ultimately depend on how well PBMC serve as surrogates for tissues that govern whole body disposition (intestine, liver etc.). Recently, cytokines were shown to affect drug transporter and CYP function in the liver (Morgan *et al.*, 2008). It is also of interest to note that cytokines have been shown to impact on levels of α1-acid glycoprotein (AAG) (Hrycaj *et al.*, 1993a,b). The protease inhibitors used in antiretroviral therapy, such as lopinavir, are highly bound to AAG in plasma (Boffito *et al.*, 2003). Therefore, cytokines may also impact upon plasma free drug concentrations that may in turn influence intracellular accumulation and hepatic extraction ratio.

In summary, we have shown the impact of cytokines on the expression and function of transporters and expression of CYPs. These proteins have wide tissue distribution and may be exposed to many cytokines. Also, the impact of cytokines on chemokine receptors has been demonstrated. The clinical relevance of these findings and mechanism by which these genes may be functionally co-regulated now warrants further study.

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## Conflict of interest

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