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Multidrug transporter activity in lymphocytes

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1 Multidrug transporters play a dual role in haematopoietic cells, mediating the efflux of xenobiotics and regulating cell migration.

2 For several reasons including the lack of specific antibodies, reports of multidrug transporter distribution on lymphocytes conflict. Murine B cells have been reported to completely lack transporter activity.

3 Through analysis of parental and 'knockout' mice we show that, contrary to previous studies, murine B and T lymphocytes possess at least three active multidrug transporters and also a hitherto unrecognised drug-specific import activity.

4 Surprisingly, the drug specificity of P-glycoprotein appears cell type dependent. The data indicate that a range of developmentally regulated, multidrug transporters can impose a barrier to treatment of immune disorders.

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Keywords: Lymphocytes; multidrug transporters; P-glycoprotein

Abbreviations: abcg2, mitoxantrone-resistance protein/breast cancer-resistance protein/bcrp/mxr/abcp1; CsA, cyclosporin A; FmC, fumitremorgin C; hprt, hypoxanthine phosphoribosyltransferase; mrp, multidrug-resistance-associated protein,abcc1; P-gp, P-glycoprotein/multidrug-resistance protein, mdr1,abcb1

Introduction

Multidrug-resistance P-glycoprotein (P-gp, mdr1, abcb1) (Gottesman & Pastan, 1993), multidrug-resistance associated protein (mrp1, abcc1) (Cole *et al*., 1992) and the mitoxantrone-resistance protein (abcg2 – breast cancer-resistance protein) (Allikmets *et al*., 1998; Doyle *et al*., 1998; Miyake *et al*., 1999) were initially recognised as serious impediments to cancer chemotherapy through their ability to eliminate drugs from cells. However, by effluxing physiological mediators such as leukotriene C4 (LTC_4) and platelet-activating factor (PAF), transporters such as mrp1 and P-gp have been implicated in the regulation of some immune cell activities, notably migration (Randolph *et al*., 1998; Prechtl *et al*., 2000; Robbiani *et al*., 2000; Frank *et al*., 2001; Honig *et al*., 2003).

Given the lack of antibodies to extracellular epitopes of murine drug transporters, analysis of fluorochrome efflux is the major indicator of their expression. However, studies of transporters expressed by lymphocytes conflict. In humans, for example, it has been suggested that P-gp is expressed by anywhere between 20 and 80% of B cells, and between 30 and 100% of $CD4^+$ T cells (Chaudhary *et al*., 1992; Drach *et al*., 1992; Klimecki *et al*., 1994; Pilarski *et al*., 1995; Ludescher *et al*., 1998) and in mice, between 15–50% of $CD4^+$ and 50–90% of $CD8^+$ cells (Bommhardt *et al*., 1994; Witkowski *et al*., 1994). Similarly, mrp1 activity has been detected either on only 3% of $CD4^+$ lymphocytes (Lohoff *et al*., 1998; Prechtl *et al*., 2000), or in the majority of T cells (albeit in the presence of the immunosuppressant FTY720) (Honig *et al*., 2003). Few studies have investigated abcg2 function in mature lymphocytes, although it has been implicated in the development of T-cell resistance to the antirheumatic drug sulfasalazine (van der Heijden *et al*., 2004). B cells have been reported to lack drug efflux activity (Bommhardt *et al*., 1994), although this observation is perhaps surprising given the reported role of P-gp and mrp1 in basal drug resistance (Allen *et al*., 2000). Several factors, such as the overlap in transporter substrate specificity, reliance on efflux of a single test substrate (such as rhodamine 123 – [Rh123]), use of potentially inhibitory antibodies (Ghetie *et al*., 1999) and variations in assays, may have contributed to the conflict in results and the failure to detect transporter activity. Further, as deficiency of mdr 1a/b may alter mrp1 activity (Honig *et al*., 2003) it is difficult to accurately assess the relative contributions of the P-gp and mrp1 to drug efflux solely from comparisons of parental and gene-targeted mice.

To assess the function of P-gp, mrp1 and abcg2 in lymphocytes and therefore their potential roles in basal drug resistance, we analysed the ability of T and B cells from parental and 'knockout' mice to efflux a wide variety of fluorochromes. The data show that drug accumulation depends on the complex, cumulative effect of multiple transporters, including an importer. Surprisingly, P-gp exhibited cell type-dependent substrate specificity. The combined activity of such transporters should be considered in developing treatment regimens.

Methods

Mice

mdr1a and *1b* mutations (Schinkel *et al*., 1997) were back-crossed for at least seven generations onto the FVB (Friend

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virus B-type susceptibility) background at Taconic farms (Germantown, U.S.A.). Mice were between 5 and 12 weeks of age. When experiments utilised FVB.mdr1a/b$^{-/-}$ mice, age-matched FVB mice were used as controls. In all other experiments, C57BL/10 or FVB mice were used. Mice had free access to food chow and tap water. Institute guidelines for the care of laboratory animals were followed.

Reverse transcription (RT)–PCR analysis of mRNA

For comparison of levels of mRNA in lymphocyte subsets, splenic B cells, CD4$^+$ T cells and CD8$^+$ T cells were sequentially selected from a cell suspension using antibody-conjugated Dynabeads (pan B [B220], CD4 and CD8, respectively), as recommended by the manufacturer (Dynal, Wirral, U.K.). RNA was extracted from separated B and T cells using RNAzol B (Biogenesis, Poole, U.K.). In all, 1 μg of total RNA from each lymphocyte subset was used in a RT reaction using first-strand cDNA Synthesis Kit, Avian Myeloblastosis Virus RT and random primers (Boehringer Mannheim, Mannheim, Germany). cDNA (50 ng) was used in each PCR reaction, containing 200 nM of each gene-specific primer, 0.25 mM dNTPs, 2 mM $MgCl_2$, 2.5 U *Taq* Polymerase (Gibco BRL, Paisley, U.K.) and 0.037 MBq (α^{32}P dCTP at 110 TBq mmol^{-1} (Amersham Pharmacia, Chalfont, U.K.)). Gene-specific primers and predicted RT–PCR product sizes were: mouse hypoxanthine phosphoribosyltransferase *(hprt)* forward 5′-GCTCGAGATGTCATGAAGGAGATG-3′ and reverse 5′-GCAGATGGCCACAGGACTAGAACA-3′, 597 bp; *mdr1a* and *mdr1b* forward 5′-AAGTGAAAAGGTT GTCCAGGA-3′; *mdr1a* reverse 5′-ATGCTTTCCACTGTG GCAGAA-3′, 390 bp; *mdr1b* reverse 5′-CTCTTATGAAT CACGTAATGC-3′, 490 bp; *mrp1* forward 5′-GCTTCTGCA GCGCTGATGGCT-3′ and reverse 5′-GTCACTGCTCTCCA GGGGCTG-3′, 706 bp; *abcg2* forward 5′-GGCTTATACGG CCAGTTCCAT-3′ and reverse 5′-CAGGTAGGCAATTGT GCGGAA-3′, 396 bp. Cycling conditions were 95°C for 5 min, 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. Aliquots were removed at every three cycles starting at 17 cycles and ending at 35 cycles. Samples were taken from the linear range of amplification to ensure relative quantitation corresponding to 20 cycles (hprt), 26 cycles (*mdr1a* and *mdr1b*), 32 cycles (*mrp1*) and 23 cycles (*abcg2*). Products from the PCR reactions were separated by denaturing polyacrylamide gel electrophoresis or by electrophoresis in 2% agarose gels, and quantified after exposure to phosphoimager screens (Amersham-Pharmacia, Chalfont, U.K.).

Quantitative real time RT–PCR from unseparated lymphocyte populations was carried out using an Opticon DNA engine continuous fluorescence detector (MJ Research Inc., Waltham, MA, U.S.A.) with Quantitect SYBR Green PCR master mix (Qiagen, Crawley, West Sussex, U.K.) and primers as above. Cycling conditions were as previously described (Yague *et al*., 2003).

Fluorochrome efflux assays

Steady-state assay of fluorochrome uptake Mesenteric lymph node cell suspensions (10^7 ml^{-1}) were incubated in the presence of fluorochrome for 30 min at 37°C in DMEM (Sigma) supplemented with 1% BSA (Sigma) in the presence of inhibitors when appropriate; 20 μM cyclosporin A (CsA, Sigma), 10–70 μM MK571 (a generous gift from Dr B. Sarkadi) or 10 μM fumitremorgin C (a generous gift from Professor S. Bates) or 0.2% DMSO as a diluent control. No significant difference in the inhibitory efficiency of MK571 was apparent across the concentrations used. To discriminate between uptake of fluorochromes by different lymphocyte subsets, CD4$^{\text{QUANTUM RED}}$, CD4$^{\text{PE}}$, CD4$^{\text{FITC}}$, CD8$^{\text{QUANTUM RED}}$, CD8$^{\text{PE}}$, CD8$^{\text{FITC}}$ (Sigma) or NK1.1$^{\text{FITC}}$ (Becton Dickinson, CA, U.S.A.) antibodies were added, as indicated, for the final 10 min. The fluorophore on antibodies used to identify CD4$^+$ and CD8$^+$ T cells varied in some experiments to avoid overlap between emission spectra of the antibody-conjugated fluorochromes and fluorescent substrates. Cells were washed with DMEM/BSA and analysed by flow cytometry (FACScalibur or FACScan using CellQuest software – Becton Dickinson). Live cells were manually gated on the basis of forward scatter and side scatter. As in preliminary experiments, over 95% of cells expressed either B (CD19)- or T-cell (CD4 or CD8) markers (not shown), lymphocytes lacking CD4 or CD8 were defined as B cells. To measure drug uptake, the following fluorochromes were used (Molecular Probes, Leiden, Netherlands, unless stated): 0.0125 μM calcein acetony methylester (AM), 0.25 μM Fluo-3 AM, 0.25 μM Fluo-4 AM, 0.5 μM BODIPY-verapamil, 0.5 μM BODIPY-prazosin, 0.02 μM BODIPY-taxol, 0.1 μM BODIPY-ceramide, 0.2 μM Fura-Red, 0.25 μM Rh123, 0.5 μM mitoxantrone (Sigma).

Statistics

Statistical significance was calculated by paired *t*-test or ANOVA as appropriate.

Results

Expression of mdr1a/b, mrp1 and abcg2 mRNA in lymphocyte subsets

In a preliminary experiment, we assessed expression of *mdr1a/b, mrp1* and *abcg2* mRNA in lymphocyte subsets, separated using antibody-coated metal beads, by means of a semiquantitative RT–PCR assay. Values were normalised with respect to *hprt*. RNA specific for each transporter was apparent in each lymphocyte subset at broadly similar levels between T and B cells (Figure 1a). No upregulation of *mrp1* or *abcg2* mRNA was apparent in lymphocytes from mdr1a/b-deficient mice (Figure 1b). That levels of transporter RNAs were similar between subsets was surprising given that murine B cells have been reported to lack P-gp activity (Bommhardt *et al*., 1994) and that mrp1 activity has been found only in CD4$^+$ T cells (Lohoff *et al*., 1998; Prechtl *et al*., 2000). Therefore, because mRNA levels do not necessarily reflect levels of functional transporter, we assessed the activity of P-gp, mrp1 and abcg2 in each lymphocyte subset.

Efflux of fluorochromes from parental and mdr1a/b-deficient lymphocytes

To assess drug transport, T cells were labelled with CD4- and CD8-specific antibodies and identified by flow cytometry. In preliminary experiments (not shown), over 95% of unlabelled population was found to be CD19$^+$ B cells. Therefore, because

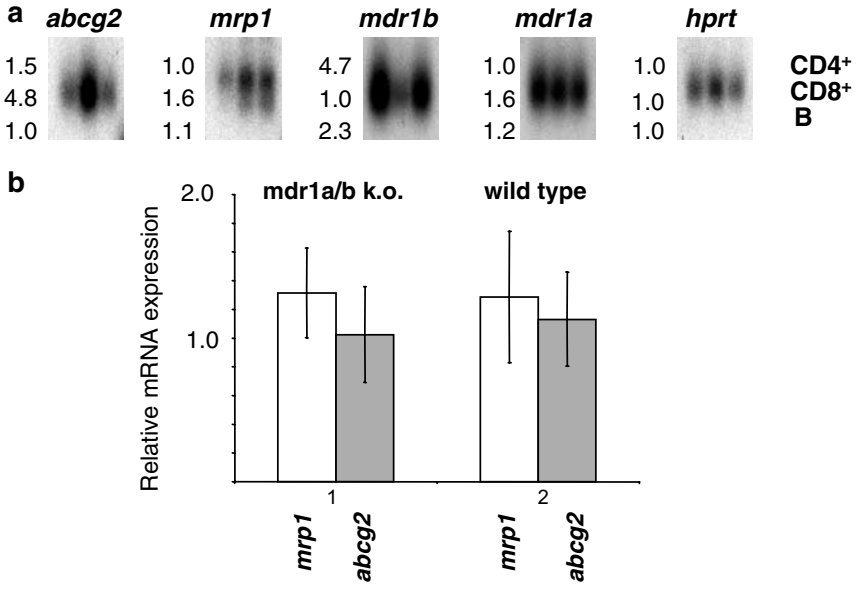

Figure 1 RNA quantitation by RT–PCR. (a) RNA was isolated from lymphocyte subpopulations, separated using antibodies linked to magnetic beads, and cDNA synthesised by reverse transcription. PCR products from the cDNA were amplified using specific primers and electrophoresed. Samples were taken from the linear range of amplification to ensure relative quantitation. PhosphorImager counts were normalised to *hprt* and relative levels of expression for each lymphocyte subset were expressed below each band (the lowest value was assigned an arbitrary value of one). (b) RNA was isolated from lymph nodes from wild type and mdr1/ab-deficient mice ($n=3$). Quantitative real-time RT–PCR was carried out using an Opticon DNA engine continuous flow detector and mRNA levels were normalised to *hprt*. Relative expression values are the means of triplicate real-time RT–PCR reactions.

anti-CD19 antibodies have been found to inhibit P-gp (Ghetie *et al*., 1999), and to keep one flow cytometer channel free for detection of fluorochrome accumulation, in subsequent experiments $CD4^-CD8^-$ cells were assumed to be B cells. Given that substrate specificities of P-gp, mrp1 and abcg2 overlap, and that uncharacterised transporters may be present, we analysed the uptake and efflux of several fluorochromes by mdr1a/$b^{+/+}$ and mdr1a/$b^{-/-}$ lymphocytes in the presence of a variety of specific and relatively broad inhibitors of multidrug transporters.

Calcein AM and Fluo-4 AM To determine which lymphocyte subsets have P-gp and/or mrp1 activity, calcein acetoxymethylester (AM) and Fluo-4 AM were used in the presence or absence of CsA (as a relatively broad multidrug transporter inhibitor). Calcein AM is a nonfluorescent substrate for both P-gp and mrp1 that enters cells and is converted into fluorescent calcein. As calcein is not a substrate for P-gp, and is a much poorer substrate for mrp1 than its AM derivative (Essodaigui *et al*., 1998), increased fluorescence upon incubation with calcein AM is inversely related to transporter activity (Homolya *et al*., 1996). Fluo-3 AM is a well-characterised substrate for P-gp and mrp1 (Homolya *et al*., 1993; Prechtl *et al*., 2000), Fluo-4 AM being a more fluorescent derivative that in preliminary studies behaved identically (not shown).

Lymphocytes were incubated with fluorochromes in the presence or absence of CsA and accumulation of fluorophore was analysed by flow cytometry. The concentrations of CsA used did not lead to measurable toxicity as indicated by propidium iodide uptake (not shown). Inhibition of P-gp and mrp1 resulted in increased uptake of calcein AM and Fluo-4 AM in B cells, $CD4^+$ and $CD8^+$ T cells by over 100-fold (as evidenced by mean fluorescence intensity) (Figure 2a). Thus, surprisingly given reports of both absence of transporter activity on B cells and paucity of mrp1 on T cells (Bommhardt *et al*., 1994; Lohoff *et al*., 1998; Prechtl *et al*., 2000), all lymphocyte subpopulations appear to express one or more efflux pumps with characteristics consistent with P-gp and/or mrp1 activity.

To assess the contribution of P-gp to the efflux of calcein AM and Fluo-4 AM, we compared fluorochrome uptake by parental and mdr1a/b-deficient lymphocytes. mdr1a/b-deficient T and B cells all showed a greater uptake of calcein AM (Figure 2b) and Fluo-4 AM (not shown) than did comparable subpopulations of parental lymphocytes. Hence, all T and B lymphocytes express functional P-gp, activity appearing slightly greater in $CD8^+$ cells than in $CD4^+$ or B cells.

mdr1a/b-deficient lymphocytes from mice retained substantial calcein AM (Figure 2c) and Fluo-4 AM (data not shown) efflux activity. As abcg2 does not transport calcein AM (Litman *et al*., 2000), it seemed likely that this was due to mrp1 activity. To test this, the mrp1 inhibitor MK571, and the abcg2 inhibitor fumitremorgin C (FmC) were used. Efflux of calcein AM by parental lymphocytes was inhibited by MK571 but not by FmC (Figure 2d). While MK571 also inhibits other members of the mrp family, to the best of our knowledge these are not expressed in lymphocytes and/or do not transport the fluorochromes used (Borst *et al*., 2004). Thus, all murine T and B cells appear to express functional P-gp and mrp1.

BODIPY-taxol The anticancer drug taxol and its conjugate BODIPY-taxol are substrates for P-gp (Binaschi *et al*., 1995; Fellner *et al*., 2002). CsA dramatically enhanced BODIPY-taxol uptake into $CD8^+$ T cells (Figure 3a), consistent with the presence of active P-gp. This was confirmed by the much

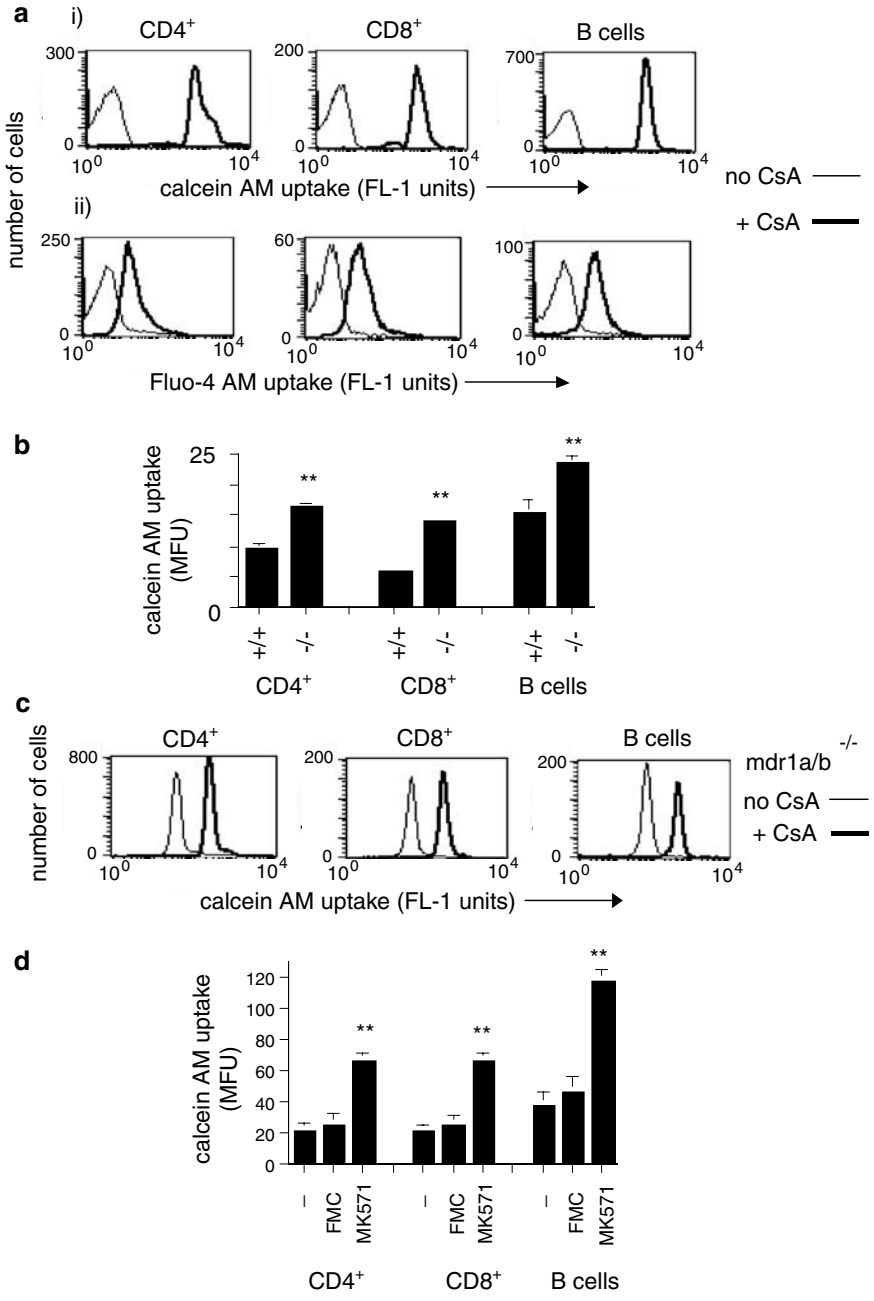

Figure 2 Expression of P-gp and mrp1 by T and B lymphocytes. The accumulation of calcein AM or Fluo-4 AM by lymphocyte subsets in the presence or absence of P-gp and mrp1 inhibitors was analysed by flow cytometry. Lymphocyte subsets were identified by staining with fluorescently conjugated antibodies. (a) Lymphocytes were incubated with calcein AM (panel i) or Fluo-4 AM (panel ii) in the presence (thick lines) or absence (thin lines) of CsA. (b) Lymphocytes from parental or mdr1a/b-deficient mice were incubated with calcein AM (**$P<0.0001$). (c) Lymphocytes isolated from mdr1a/b-deficient mice were incubated with calcein AM in the presence (thick lines) or absence (thin lines) of CsA. (d) Lymphocytes from mdr1a/b-deficient mice were incubated with calcein AM in the presence or absence of either the mrp-specific inhibitor MK571 or the abcg2 inhibitor FmC (**$P<0.00001$). Data are representative of at least three independent experiments.

greater accumulation of BODIPY-taxol by mdr1a/b-deficient $CD8^+$ T lymphocytes than by cells from parental mice (Figure 3b). Little or no CsA-inhibitable transport of BODIPY-taxol was detected in mdr1a/b-deficient lymphocytes (Figure 3c), consistent with the lack of specificity of mrp1 and abcg2 for this substrate (Litman *et al*., 2000). Moreover, the negligible effect of CsA on BODIPY-taxol uptake in mdr1a/b-deficient cells indicates that, at the concentrations used, any effects of this inhibitor on fluorochrome uptake were not due to toxicity. In our hands, the pattern of BODIPY-taxol uptake resembled that of Rh123 (not shown).

However, BODIPY-taxol was unexpectedly (given the presence of P-gp demonstrated above) found to be strongly accumulated by B cells, and uptake being affected little by CsA. Similarly, accumulation was only slightly greater in B cells from parental than from mdr1a/b-deficient mice

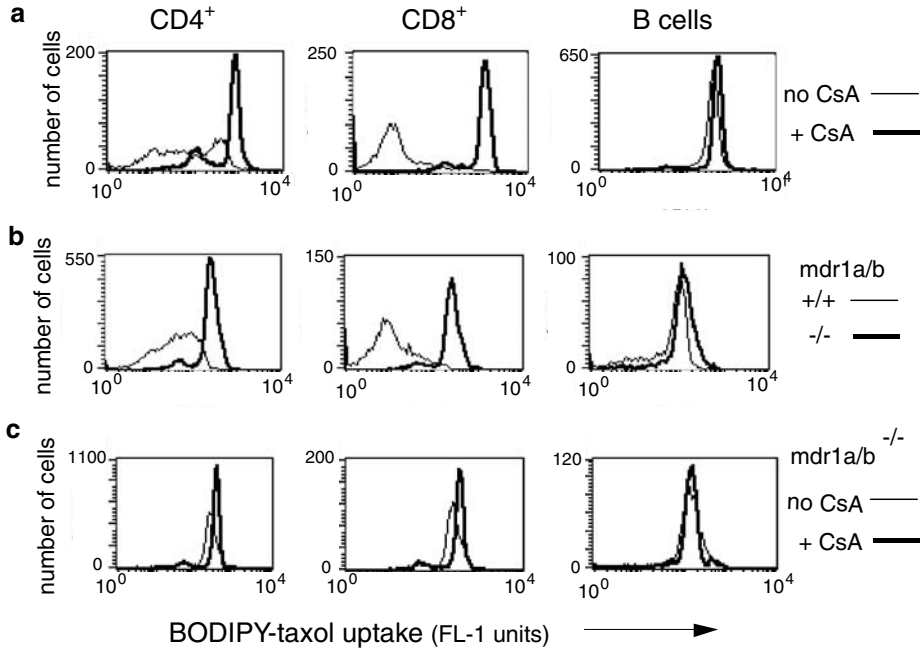

Figure 3 P-gp-dependent efflux of BODIPY-taxol. Lymphocytes were labelled with fluorescently conjugated antibodies and gated by flow cytometry. (a) Cells were incubated with BODIPY-taxol (BT) in the presence (thick lines) or absence (thin lines) of CsA. (b) Lymphocytes from parental (thin lines) or mdr1a/b-deficient (thick lines) mice were incubated with BT. (c) Lymphocytes from mdr1a/b-deficient mice were incubated with BT in the presence (thick lines) or absence (thin lines) of CsA. Data are representative of at least three independent experiments.

(Figure 3b). Thus despite the presence of P-gp, BODIPY-taxol is very poorly effluxed from B cells (Figure 3a). The simplest interpretation of this finding is that the efficiency with which P-gp transports individual drugs differs between cell types, and that BODIPY-taxol is a relatively poor substrate in B cells.

$CD4^+$ T cells were intermediate (between $CD8^+$ T cells and B cells) in their ability to efflux BODIPY-taxol. Although there was some P-gp-dependent BODIPY-taxol efflux (Figure 3a, b), it was much less than expected given the similarity of P-gp activity in $CD4^+$ and $CD8^+$ cells towards calcein AM and Fluo-4 AM. Unlike calcein AM and Fluo-4 AM, uptake of BODIPY-taxol by $CD4^+$ cells was also markedly heterogeneous (Figure 3a, b).

BODIPY-verapamil and BODIPY-prazosin BODIPY-verapamil is a substrate for P-gp (Dey *et al*., 1999) and BODIPY-prazosin for both P-gp (Lee *et al*., 1998) and abcg2 (Litman *et al*., 2000). In $CD8^+$ T cells, accumulation of the two drugs was increased slightly in mdr1a/b-deficient cells compared with wild-type controls (Figure 4a), indicating a small amount of P-gp-dependent efflux, albeit restricted to $CD8^+$ cells. The mrp1 inhibitor MK571 (but not the abcg2 inhibitor FmC) enhanced BODIPY-verapamil uptake by mdr1a/$b^{-/-}$ lymphocytes, suggesting that this drug is also a substrate for mrp1 (see below).

Surprisingly, despite the P-gp and mrp1-dependent efflux of BODIPY-prazosin and BODIPY-verapamil, CsA did not increase, but instead marginally decreased fluorochrome uptake by a proportion of cells (Figure 4b for BODIPY-verapamil; data not shown for BODIPY-prazosin). We therefore examined the effects of CsA on BODIPY-verapamil accumulation in the absence of P-gp and mrp1 activities (mdr1a/$b^{-/-}$ lymphocytes in the presence of MK571). Under these conditions, CsA markedly decreased BODIPY-verapamil uptake (Figure 4c). The simplest interpretation of these data is that CsA inhibits an undefined drug importer. To exclude the possibility that the CsA-dependent decrease in BODIPY-verapamil uptake was due to adverse effects of these drugs on lymphocytes, we showed that in the same aliquot of cells, incubation with CsA resulted both in increased accumulation of Fura Red (which emits in FL-2 and can be used simultaneously with BODIPY-conjugates) and in decreased accumulation of BODIPY-verapamil (Figure 4d).

Accumulation of BODIPY-verapamil and BODIPY-prazosin by lymphocytes is thus determined by the overlapping activities of at least three transporters, namely P-gp and mrp1 mediating export, and an uncharacterised importer.

Mitoxantrone ABCG2 is a drug exporter (Allikmets *et al*., 1998; Doyle *et al*., 1998; Miyake *et al*., 1999) first detected in breast tissue. To assess the potential activity of abcg2 in lymphocytes, we utilised mitoxantrone, a substrate for ABCG2 (Litman *et al*., 2000) and P-gp (Allikmets *et al*., 1998; Doyle *et al*., 1998; Miyake *et al*., 1999), and the ABCG2 inhibitor FmC. Mitoxantrone is not a substrate of mrp1 (Litman *et al*., 2000).

In mdr1a/b-deficient T cells, CsA-inhibitable efflux of mitoxantrone (Figure 5a) indicated a role for a transporter other than P-gp. Both the abcg2 inhibitor FmC and the mrp1 inhibitor MK571 equivalently, but not additively, increased the uptake of mitoxantrone in mdr1a/b-deficient cells (Figure 5b), suggesting that MK571 inhibits abcg2 as well as mrp1. Consistent with *abcg2* mRNA data (Zhou *et al*., 2001), we found that abcg2 activity is highest in NK cells, although activity in other lymphocytes, particularly $CD4^+$ T cells, was significant (Figure 5c). Accumulation

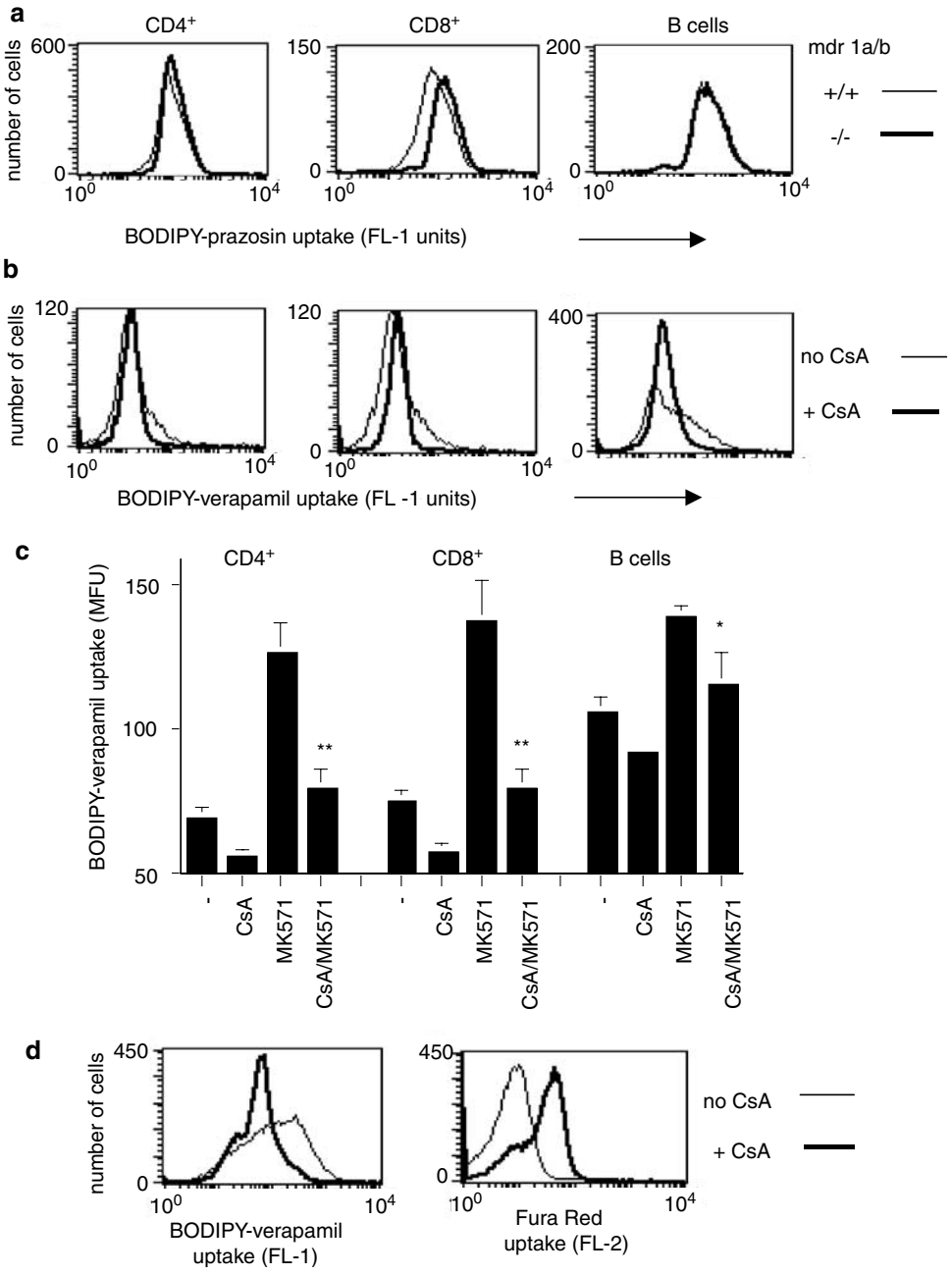

Figure 4 Uptake and efflux of BODIPY-verapamil and BODIPY-prazosin. The accumulation of BODIPY-verapamil and BODIPY-prazosin by lymphocyte subsets in the presence/absence of inhibitors of P-gp and mrp1 was analysed by flow cytometry. Lymphocyte subsets were gated after labelling with fluorescently conjugated antibodies. (a) Lymphocytes from parental (thin lines) or mdr1a/b-deficient (thick lines) mice were incubated with BODIPY-prazosin. (b) Lymphocytes (parental mice) were incubated with BODIPY-verapamil in the presence (thick lines) or absence (thin lines) of CsA. (c) Lymphocytes from mdr1a/b-deficient mice were incubated with BODIPY-verapamil alone, or in the presence of CsA, the mrp1 inhibitor MK571 or CsA and MK571. (difference in MFU in presence MK571 vs MK571/CsA – $*P<0.02$; $**P<0.001$) (d) Lymphocytes were incubated with both BODIPY-verapamil and Fura-red in the presence (thick lines) or absence (thin lines) of CsA. The CsA-inhibitable import of BODIPY-verapamil (left panel) and efflux of Fura-red (right panel) occurring in the same cell population are distinguishable due to the different emission spectra of the two fluorochromes. Data from (a, b) are representative of at least three independent experiments, and (c, d) of two experiments.

of mitoxantrone was slightly greater in mdr1a/b-deficient $CD8^+$ and B lymphocytes than those from parental controls, confirming that it is also a substrate for P-gp (Figure 5d). The relatively high abcg2 activity in $CD4^+$ T cells may mask P-gp-dependent efflux of mitoxantrone in this population.

Discussion

Lymphocyte multidrug transporters impose barriers to treatment of cancer and potentially many other immune-related disorders (Kim *et al*., 1998; Jansen *et al*., 2003). However, conflicting results have complicated assessment of the likely

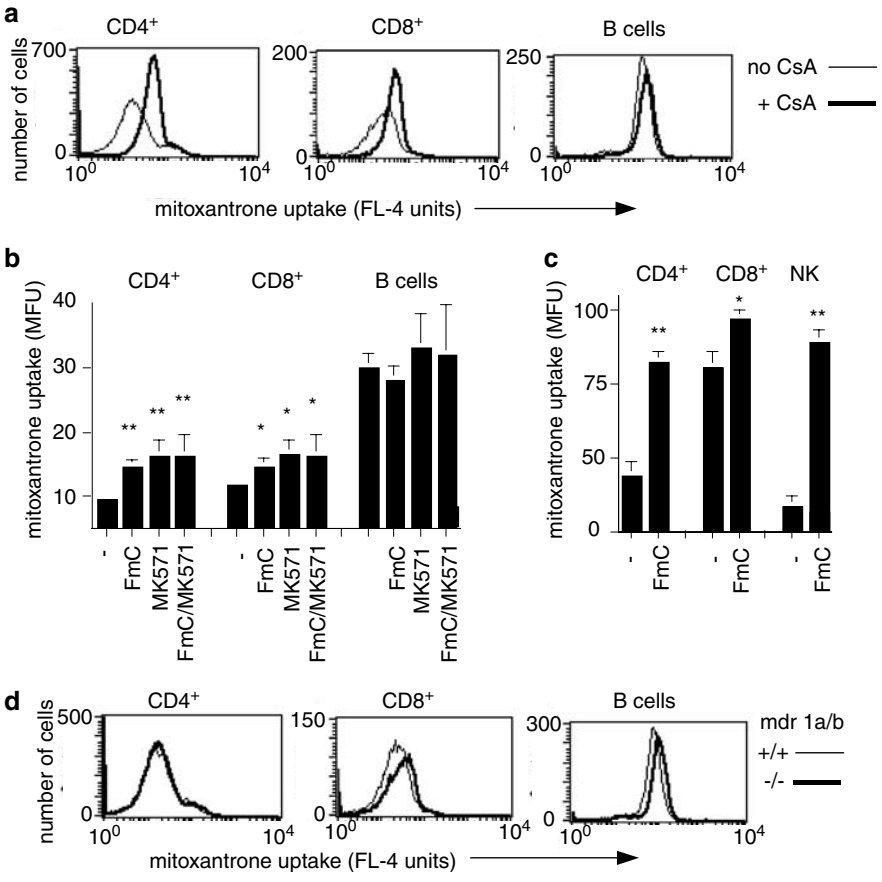

Figure 5 Efflux of mitoxantrone by lymphocytes. Lymphocyte subsets were distinguished by staining with fluorescently conjugated antibodies to CD4 and CD8. (a) Lymphocytes from mdr1a/b-deficient mice were incubated with mitoxantrone in the presence (thick lines) or absence (thin lines) of CsA. (b) Lymphocytes from mdr1a/b-deficient mice were incubated with mitoxantrone alone, or in the presence of the abcg2 inhibitor FmC, the mrp1 inhibitor MK571 or FmC and MK571 ($*P<0.01$; $**P<0.001$). (c) Lymphocytes from mdr1a/b-deficient mice were incubated with mitoxantrone alone or in the presence of FmC ($*P<0.01$; $**P<0.0001$). (d) Lymphocytes from parental (thin lines) and mdr1a/b-deficient (thick lines) mice were incubated with mitoxantrone. Data (a, d) are representative of at least three independent experiments.

role of multidrug transporters in lymphocyte drug uptake (see Introduction). There are probably several reasons for such inconsistencies, including the overlap of transporter substrates; the use of assays that rely on high, potentially toxic, initial substrate loading; inhibition of P-gp by anti-B-cell antibodies (Ghetie *et al*., 1999), reliance on single fluorochromes as readouts for transporter activity and functional interactions between transporters (Honig *et al*., 2003). It is notable in our study that inferring patterns of P-gp expression simply on the basis of differential fluorochrome uptake by mdr1a/b-deficient and parental lymphocytes would in each case lead to different conclusions if one studied uptake of BODIPY-taxol, calcein AM, BODIPY-prazosin or mitoxantrone independently.

The most significant conclusion of our study is that all normal lymphocytes possess potent transport activity, mediating both efflux and import. Indeed, use of CsA to block both P-gp and mrp1 activity increased calcein AM uptake by over 100-fold. Using mdr1a/b-deficient cells, we were able to demonstrate active P-gp on all T and B cells. Previously, others have reported that P-gp is present only on $CD8^+$ T cells, a minority of $CD4^+$ T cells, and not B cells (Witkowski & Miller, 1993; Bommhardt *et al*., 1994; Witkowski *et al*., 1994). These studies, however, inferred P-gp expression from the efflux of a single fluorochrome and, as we have shown, this may be misleading. Indeed, for none of the fluorochromes used was P-gp the only factor influencing uptake. Studies involving gene-targeted mice must be treated with caution. Indeed, it has been reported that, at least in some systems, mdr1a/b deficiency inhibits the activity of mrp1 (Honig *et al*., 2003). Nevertheless, the results we obtained from the use of gene-targeted mice were consistent with those derived from the use of specific inhibitors and thus with the expression of multiple transporters in all cell types.

A surprising finding was that P-gp-dependent efflux of calcein AM and Fluo-4 AM by B and T cells differed from that of BODIPY-taxol. Thus, the efficiency with which P-gp transports specific drugs, and by inference physiologic substrates, is cell type dependent. It is unlikely, although not impossible, that such differences are due to differences in relative expression of mdr1a and mdr1b as these isoforms have been found to have similar specificities (Taylor *et al*., 1999). Moreover, T and B cells in our hands appear to express broadly similar levels of *mdr1a/b* RNA. Rather, we suggest that as the lymphocyte plasma membrane is a highly heterogeneous structure, it is unlikely that drugs differing in electrochemical properties partition equivalently, for example into rafts. Hence, as plasma membrane lipid composition differs between lymphocyte populations (Dillon *et al*., 2000),

drug distribution will differ between cell types, affecting the probability of their interaction with P-gp. It is also established that drug binding to P-gp is affected by lipid composition (Romsicki & Sharom, 1999). We suggest therefore that lymphocyte plasma membranes differ such that compounds like taxol and Rh123 interact efficiently with P-gp in $CD8^+$ cells but not B cells, while other substrates (such as calcein AM) distribute such that interaction with P-gp is similar in all lymphocytes.

Using mdr1a/b-deficient mice we demonstrated that all lymphocytes exhibit activity consistent with mrp1 function. Previous studies have been somewhat contradictory, with mrp1 activity found by one group on only 3% of $CD4^+$ T cells (Lohoff *et al*., 1998), while others have reported broad (unquantified) T-cell activity (Honig *et al*., 2003) (at least in the presence of the synthetic sphingosine FTY720). Detection of mrp1 function in our study may have been facilitated by the use of Fluo-4 AM, which is a more fluorescent analogue of Fluo-3 AM used elsewhere (Lohoff *et al*., 1998; Honig *et al*., 2003).

Abcg2, which has been shown to be expressed at high level in bone marrow stem cells and NK cells (Zhou *et al*., 2001), can transport sterols and steroids (Janvilisri *et al*., 2003) and has been implicated in resistance to sulfasalazine (van der Heijden *et al*., 2004). Abcg2 function was apparent in T cells but not B cells, although the inability to efflux a single substrate (as for P-gp) does not necessarily imply absence of functional protein. That B cells accumulated more mitoxantrone than T cells even in the presence of FmC and MK571 suggests B cell-specific mitoxantrone importers and/or T-cell-specific exporters remain to be characterised.

Analysis of BODIPY-verapamil and BODIPY-prazosin transport revealed an unexpected drug import activity in lymphocytes. The presence of a drug importer was initially suggested by the finding that CsA decreased (albeit weakly) uptake of BODIPY-verapamil and BODIPY-prazosin. The true potency of the drug importer was apparent when mrp1 activity was inhibited in mdr1a/b-deficient lymphocytes using MK571. In these circumstances, CsA strongly reduced BODIPY-verapamil and BODIPY-prazosin uptake. Accumulation of BODIPY-verapamil and BODIPY-prazosin, therefore, reflects a balance between the activities of P-gp and abcg2 and mrp1 (mediating export) and a novel drug importer. We do not yet know the identity or normal biological role of the importer.

If replicated in human cells, our data have several implications for clinical practice. First, the potential benefit of inhibiting multidrug-resistance proteins in drug treatment of a wide range of immune-related conditions is apparent. As hydrophobic cytotoxic drugs are frequently used in the treatment of immune-related conditions from transplant rejection to autoimmune disease, identification of transporters may be central to the design of drug treatments. For example, as P-gp and mrp1 can efflux HIV protease inhibitors, their expression on $CD4^+$ T cells may impede treatment of AIDS (Kim *et al*., 1998; Fellay *et al*., 2002; Dallas *et al*., 2004). Second, caution is required in clinical trials of multidrug-resistance reversal in cancer as treatment with cytotoxic drugs in combination with a reversal agent is likely to exacerbate immune system damage. Finally, the efficiency with which P-gp transports drugs appears cell type dependent. Our findings therefore illustrate the complex interaction of multidrug transporters, their substrates and the plasma membrane in the transport of drugs into lymphoid cells.

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