

High affinity capture and concentration of quinacrine in polymorphonuclear **neutrophils** via vacuolar ATPase-mediated ion trapping: comparison with other peripheral blood leukocytes and implications for the distribution of cationic drugs

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## **Abstract**

Many cationic drugs are concentrated in acidic cell compartments due to low retro-diffusion of the protonated molecule (ion trapping), with an ensuing vacuolar and autophagic cytopathology. In solid tissues, there is evidence that phagocytic cells, e.g., histiocytes, preferentially concentrate cationic drugs. We hypothesized that peripheral blood leukocytes could differentially take up a fluorescent model cation, quinacrine, depending on their phagocytic competence. Quinacrine transport parameters were determined in purified or total leukocyte suspensions at 37°C. Purified polymorphonuclear leukocytes (PMNLs, essentially neutrophils) exhibited a quinacrine uptake velocity inferior to that of lymphocytes, but a consistently higher affinity (apparent  $K_M$  1.1 vs. 6.3  $\mu M$ , respectively). However, the vacuolar (V)-ATPase inhibitor bafilomycin A1 prevented quinacrine transport or initiated its release in either cell type. PMNLs capture most of the quinacrine added at low concentrations to fresh peripheral blood leukocytes compared with lymphocytes and monocytes (cytofluorometry). Accumulation of the autophagy marker LC3-II occurred rapidly and at low drug concentrations in quinacrine-treated PMNLs (significant at  $\geq 2.5$   $\mu M$ ,  $\geq 2$  h). Lymphocytes contained more LAMP1 than PMNLs, suggesting that the mass of lysosomes and late endosomes is a determinant of quinacrine uptake  $V_{max}$ . PMNLs, however, exhibited the highest capacity for pinocytosis (uptake of fluorescent dextran into endosomes). The selectivity of quinacrine distribution in peripheral blood leukocytes may be determined by the collaboration of a non-concentrating plasma membrane transport mechanism, tentatively identified as pinocytosis in PMNLs, with V-ATPase-mediated concentration. Intracellular reservoirs of cationic drugs are a potential source of toxicity (e.g., loss of lysosomal function in phagocytes).

**Keywords:** quinacrine; polymorphonuclear leukocytes; lymphocytes; drug transport; lysosomotropic drugs; ion trapping; vacuolar ATPase; macroautophagy.

## Introduction

Many cationic drugs are concentrated in acidic cell compartments due to low retro-diffusion of the protonated molecule (ion trapping; reviewed by De Duve et al., 1974; Kaufmann and Krise, 2007; Marceau et al., 2012). In examined cultured cells, the driving force of this pseudo-transport is provided by vacuolar (V)-ATPase, a proton pump expressed in the trans-Golgi and derived organelles (endosomes, lysosomes, secretory granules). Thus, specific vacuolar (V)-ATPase inhibitors such as bafilomycin A1 have been extensively used to document the cellular capture and retention of amines, such as triethylamine (Et<sub>3</sub>N; Morissette et al., 2005) and drugs from various therapeutic classes that can be considered to be substituted forms of this tertiary amine: in increasing order of lipophilicity, procainamide (Morissette et al., 2008), lidocaine (Bawolak et al., 2010), chloroquine (Zheng et al., 2011), quinacrine (Marceau et al., 2009; 2013) and amiodarone (Stadler et al., 2008; Morissette et al., 2009). The threshold concentration for inducing the retention of amines and the characteristic vacuolar morphology that derives from it decreases with increased lipophilicity because the first step of ion trapping is believed to be simple diffusion of the uncharged form through the plasma and vacuolar membranes. Further, it has been consistently observed that the vesicles become autophagosomes, possibly due to the fact that lysosomal enzymes are inhibited at the higher pH determined by the massive trapping of basic molecules; thus, cellular/granular accumulation of macroautophagic effectors, such as microtubule-associated protein light chain 3 (LC3), may indicate the inhibition of the basal autophagic flux rather than the triggering of autophagy (reviewed by Marceau et al., 2012). Morphologically, the amine-induced giant vacuoles evolve toward multilamellar inclusions as a function of time, a cytopathology called phospholipidosis.

Several lines of evidence indicate that cationic drugs may exhibit a tropism for phagocytic leukocytes. In the Et<sub>3</sub>N series, for instance, the lipophilic anti-arrhythmic drug amiodarone and its des-ethyl metabolite are well known to be concentrated by peripheral blood neutrophils and induce inclusion bodies in these cells (Somani et al., 1986; Adams et al., 1986). Further, the abnormal skin pigmentation seen in many patients under chronic amiodarone therapy is a drug storage disease that essentially concerns dermal histiocytes (Delage et al., 1975; Ammoury et al., 2008). Corneal opacities and hepatic and lung inflammation are also probably related to tissue

accumulation of amiodarone (Vassalo and Trohman, 2007). This drug was effectively taken up much more efficiently *in vitro* by human adherent macrophages vs. cell of mesenchymal origin, vascular smooth muscle cells (Morissette et al., 2009). Systemically administered amiodarone is rapidly concentrated in the liver and lungs of rats, but not efficiently in fat (Wyss et al., 1990), supporting the hypothesis that there may be a tropism of such drugs for macrophage-rich tissues. An alternate lipophilic substituted Et<sub>3</sub>N formerly used as an antiprotozoal drug, quinacrine, also concentrates in the liver, lungs and spleen *in vivo* and induces side effects dependent on tissue accumulation (skin and oral mucosa discoloration, possible role in a reversible hepatitis; Ehsanian et al., 2011). It thus seems possible that phagocytes accumulate cationic drugs more effectively than other cell types, with possible effects on cell functions (e.g., autophagy, alteration of hydrolase activities; Baritussio et al., 2001) and, ultimately, on immunity.

Neutrophils are the only terminally differentiated phagocytes in peripheral blood. We hypothesized that peripheral blood leukocytes could differentially concentrate a fluorescent model cation, quinacrine, depending on their phagocytic competence, and addressed the mechanisms of the capture and retention of the drug, including but not limited to V-ATPase-mediated ion trapping. Quinacrine was used in these studies since it exhibits an intrinsic strong green fluorescence that is well suited for morphological and cell transport studies (Marceau et al., 2009; 2013).

## Methods

**Drugs.** Bafilomycin A1 was purchased from LC Laboratories (Woburn, MA) and all other drugs, from Sigma-Aldrich (St. Louis, MO).

**Cells.** The institutional research ethics board approved the anonymous use of human citrated blood from healthy volunteers to obtain leukocytes. PMNLs (essentially neutrophils) and mononuclear cells were prepared according to Fernandes et al. (2006), with some modifications. Briefly, after sedimentation of red blood cells in 2% dextran, PMNLs were aseptically purified by centrifugation on Ficoll-Paque cushions. Contaminating erythrocytes were removed by hypotonic lysis and PMNLs were resuspended ( $10^6$ /ml) in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). Mononuclear cells were also recovered in some experiments, washed twice in Hank's balanced salt solution without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (HBSS), resuspended in RPMI 1640 supplemented with 10 % FBS, plated in plastic 75-cm<sup>2</sup> flasks and incubated for 2 h at 37°C in a humidified atmosphere containing 5 % CO<sub>2</sub>. The non-adherent cells, predominantly lymphocytes, were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% FBS and used in comparative transport and microscopy experiments.

**Quinacrine transport.** The uptake of quinacrine was established by a variation of a technique previously applied to quantify cell uptake of this drug in adherent smooth muscle cells (Marceau et al., 2009): test drugs were added to 1 ml cell suspensions ( $10^6$  cells) based on the serum-containing culture media described above according to various schemes and time frames. The cells, protected from light, were incubated under agitation at 37°C (Thermomixer, Eppendorf), rapidly centrifuged (12,500 rpm, 30 sec), washed with 1 ml of phosphate-buffered saline, pH 7.4, at room temperature, recentrifuged and the final pellet was dissolved in 1 ml of 1 N NaOH. Quinacrine was analyzed in the NaOH extract using a SLM-AMINCO-Bowman Series 2 luminescence spectrophotometer against a standard curve of the authentic drug dissolved in NaOH 1 N (excitation 414 nm, emission 501 nm). Control fluorescence from extracts of untreated cells was systematically verified, of small magnitude and subtracted from experimental values.

**Microscopy and cytofluorometry.** Epifluorescence microscopy was used to ascertain the cellular uptake and subcellular distribution of quinacrine in PMNLs or lymphocytes purified as described above and maintained in the indicated culture medium. In addition, total peripheral blood leukocytes, prepared from citrated blood submitted to hypotonic lysis and resuspended in HBSS, were also incubated with quinacrine (30 min), to monitor the cell type into which the associated green fluorescence was associated as a function of drug concentration. The cells were spun, resuspended in HBSS and the drug uptake in cell populations assessed using the BD SORP LSR II cell analyzer (BD Biosciences, Franklin Lakes, NJ; green fluorescence). The results were analyzed using the BD FACS DIVA software.

Fluid phase endocytosis (pinocytosis) was evaluated in pure population of leukocytes or myeloid cell lines by measuring the uptake of dextran (40 kDa) conjugated to rhodamine B (Sigma-Aldrich; final concentration in HBSS 1 mg/ml; adapted from Pataki et al., 1995). After a 30-min incubation at 37°C, the cells were centrifuged and washed prior to being photographed (green fluorescence and transmission, 1000×). Photoshop software (version 6, Adobe Systems, Mountain View, CA, USA) was used to manually delineate each cell in the photographic record of fluorescence from which the median pixel intensity was derived and statistically analyzed. A variation of the technique involved another conjugated dextran taken up by cells via pinocytosis: LysoSensor Yellow/Blue dextran (Invitrogen) contains a fluorophore that is pH indicator of the endocytic/lysosomal pathway (yellowish at low pH; Diwu et al., 1999; Lee et al., 2010). Leukocytes suspensions ( $10^6$ /ml) in their respective culture medium were treated with 1 mg/ml of LysoSensor Dextran for 1 h; then the washed cells were either observed in microscopy (under ultraviolet excitation using an electronic camera that covered all visible wavelengths) or assessed using cytofluorometry (excitation at 355 nm, readings at both 440 and 535 nm wavelengths; Lee et al., 2010).

**Immunoblots.** The effect of drugs (0-4 h treatments) on autophagic signaling was tested in PMNLs maintained in DMEM + 10% FBS ( $8 \times 10^6$  cells per 25-cm<sup>2</sup> flasks). After treatment, the cells were centrifuged, washed in HBSS, respun, resuspended in sample buffer and boiled as described (Marois et al., 2011). Cells extracts equivalent to  $1.6 \times 10^6$  cells per lane were run on

12% SDS-PAGE and transferred to PVDF membranes. Anti-human LC3B rabbit polyclonal antibodies (Novus; dilution 1:3000) were used to detect the cytosolic form LC3-I and processed particulate form LC3-II in PMNLs treated with quinacrine for 0-4 h (Morissette et al., 2008). Equal protein loading was verified by immunoblotting with an anti- $\beta$ -actin antibody. The expression of lysosome associated membrane protein 1 (LAMP1) was compared in leukocyte populations (cell extracts equivalent to  $0.8 \times 10^6$  cells per lane run on a 9% SDS-PAGE) using monoclonal antibodies from Iowa Developmental Hybridoma Bank (1:1000). The results were corroborated with alternate rabbit polyclonal antibodies (H-228, Santa Cruz Biotechnology). The staining was detected with the appropriate horseradish peroxidase-conjugated secondary antibodies and a luminescent substrate used as directed (Western Lightning, PerkinElmer).

**Data analysis.** Numerical values are means  $\pm$  SEM. The transport kinetics curves were fitted by the nonlinear regression method to the Michaelis-Menten equation the derived  $V_{\max}$  and  $K_M$  and their respective SEM were obtained using Prism 5.0 (GraphPad Software, San Diego, CA). The effect of bafilomycin A1 treatment of the cell uptake of quinacrine was assessed using Student's t test for paired samples. The uptake of quinacrine by 3 types of unfractionated leukocytes was compared with ANOVA followed by Tukey-Kramer multiple comparison test for each tested quinacrine concentration. Densitometric values from LC3 immunoblots were compared with ANOVA followed by Dunnett's test (comparison with a common control). The numerical data derived from the microscopic analysis of fluorescent dextran uptake were averaged for many cells and the effects of cell type was evaluated using the Mann-Whitney test (appropriate for the non-normal distributions found; InStat 3.05 program, GraphPad Software).

## Results

### *Quinacrine transport in purified peripheral blood PMNL and lymphocytes*

Quinacrine transport parameters were determined in purified leukocyte suspensions at 37°C for a standard period of 30 min (Fig. 1A, B). Purified PMNLs exhibited a quinacrine uptake velocity inferior to that of lymphocytes ( $V_{\max}$   $3.71 \pm 0.50$  vs.  $12.40 \pm 3.47$ , respectively; Fig. 1A, non-overlapping 95% confidence limits reported in Table 1), but a consistently higher affinity (lower  $K_M$  value). To reduce the variability, affinity values were best estimated using normalized transport data expressed as a percent of the intensity recorded at the highest tested quinacrine concentration (Fig. 1B, reported as corrected  $K_M$  in Table 1). Thus, the corrected  $K_M$  value of  $1.14 \mu\text{M}$  has been calculated for PMNLs and that of  $6.32 \mu\text{M}$  for lymphocytes with non-overlapping 95% confidence limits.

The fluorometric measurement of quinacrine uptake was exploited in both leukocyte subtypes to test whether V-ATPase activity drives the transport, as observed in other human cell types (Marceau et al., 2009; 2013). A fixed quinacrine concentration superior to the  $K_M$  values for each cell type was exploited in these experiments ( $2.5$  and  $10 \mu\text{M}$  for PMNLs and lymphocytes, respectively). Pretreatment of cells with the specific V-ATPase inhibitor bafilomycin A1 profoundly and significantly abated quinacrine uptake in PMNLs and lymphocytes (Fig. 1C, D). Further, bafilomycin addition to loaded cells (30 min) initiated a significant quinacrine release from both leukocyte subtypes during a subsequent 30-min incubation period, indicating that the retention of the cationic drug requires the V-ATPase activity. While V-ATPase is mainly vacuolar and intracellular in most cell types, the translocation of V-ATPase from specific vesicles to the cells surface has been observed in response to N-formyl-Met-Leu-Phe (fMLF) stimulation in human neutrophils, which acquire the capacity to secrete protons in the extracellular fluid (Nanda et al., 1996). Thus, this form of stimulation may redistribute the proton pump in such a way that the cell uptake of quinacrine is reduced if the mobilized vacuoles are responsible for a large fraction of it. However, acute fMLF stimulation, applied prior to transport measurements, did not modify quinacrine uptake in PMNLs (Fig. 1E, transport parameters reported in Table 1, including corrected  $K_M$  values).



While the classical model of cation trapping involves the simple diffusion of drugs through the plasma membrane (De Duve et al., 1974), the possible contribution of transporters at this level has been addressed using an inhibitor of multiple organic cation transporters (OCTs), decynium-22 (Hayer-Zillgen et al., 2002). This transport inhibitor did not significantly alter the increase in quinacrine concentration in PMNLs (Fig. 1F, Table 1). Similar observations were made with prazosin, used at a concentration of 10  $\mu\text{M}$ , which also failed to modify the uptake parameters of quinacrine (data not shown). Prazosin is an alternate inhibitor of OCT-1 ( $\text{IC}_{50}$  1.8  $\mu\text{M}$ ; Hayer-Zillgen et al., 2002), a transporter reportedly present in neutrophils (Engler et al., 2011).

Overall, results of comparative experiments in the two freshly isolated and non-adherent leucocyte populations support that quantitative differences exist between them in the transport of quinacrine. On the other hand, commonalities with previously examined cultured cell types include dependence of the uptake and retention on V-ATPase and absence of evidence for an identifiable transporter at the plasma membrane.

#### *Subcellular distribution of quinacrine in purified peripheral blood PMNL and lymphocytes*

The morphologic correlates of the transport experiments are presented in Fig. 2 and 3. In both PMNLs and lymphocytes, the green fluorescence associated with quinacrine was seen in perinuclear granules of various sizes (Fig. 2). It was confirmed that PMNLs take up the drug at lower concentrations ( $\geq 20$  nM) than lymphocytes ( $\geq 500$  nM). Bafilomycin A1 pretreatment abated the uptake of quinacrine in both cell types, based on the intensity of the granular fluorescence (Fig. 2). Three-dimensional reconstitutions of single representative PMNLs treated for 30 min with quinacrine (1 or 5  $\mu\text{M}$ ) are presented in Fig. 3. In these representations, the green fluorescence of single cells is shown with an increasing number of confocal planes removed from the cell top in successive images. It can be seen that the fluorescence has a granular-vacuolar distribution, and that some of the largest and brightest vacuoles have a peri-nuclear location. The multilobed nuclei themselves are seen in negative, a significant finding because quinacrine is reported to bind DNA at certain concentrations (Ehsanian et al., 2011; Gasparian et al., 2012). As

in other and previously examined cell types, the subcellular localization of quinacrine is granular-vacuolar and extranuclear, implying an extreme concentration capacity of the concerned organelles *vs.* the extracellular concentration of the drug.

#### *Quinacrine uptake in mixed peripheral blood leukocytes*

Incubating total peripheral blood leukocytes resuspended in HBSS with various concentrations of quinacrine is a simulation of a drug dosing situation in which the represented cell types can compete with each other in proportion of their relative abundance and drug uptake affinity. Cytofluorometric results corroborated the preferential uptake of the model drug by PMNLs *vs.* monocytes and lymphocytes (identified by their differential forward and side scatter properties by cytofluorometry, Fig. 4). This is notably documented in Fig. 4B where the median green fluorescence of cells is plotted as a function of quinacrine concentration: PMNLs captured significantly more of the drug at all tested concentrations (5 nM-1  $\mu$ M) relative to the two other cell types. Interestingly, monocytes are not more active in this respect than lymphocytes. This quantitative evaluation shows that the uptake of quinacrine is very selective for PMNLs at relevant therapeutic concentrations for a malaria regimen (peak concentration of 320 nM, corresponding to ~50 nM of unbound quinacrine in plasma; Ehsanian et al., 2011).

#### *Macroautophagic signalling induced by quinacrine in PMNLs*

Macroautophagic signalling (accumulation of lipidated and membrane-bound LC3 II, an autophagy effector) and labelling of the large vacuoles by LC3 were consistently observed in various cultured cell types that concentrate amines via ion trapping (Marceau et al., 2012). As in smooth muscle cells, it was observed that LC3 II, and total LC3 (I + II) accumulate in PMNLs for 4 h with a quinacrine concentration superior or equal to 2.5  $\mu$ M, and that this secondary response is statistically significant for an incubation period as short as 2 h (Fig. 5). Simply incubating the PMNLs at 37°C for 1-4 h did not induce significant LC3 accumulation *vs.* non-incubated cells.

Bafilomycin A1 in this series of experiments (Fig. 5B) is used as a positive control that inhibits the autophagic digestion in various cell types, without the production of giant vacuoles (Boya et al., 2005; Marceau et al., 2012). While the threshold concentration of quinacrine for inducing

LC3 conversion is comparatively high, the drug is known to considerably accumulate over time in tissues and cells during chronic dosing (Ehsanian et al., 2011).

*Possible contributing factors accounting to the differences of quinacrine transport between PMNLs and lymphocytes*

A possible reason for the differences in transport parameters between PMNLs and lymphocytes is a variable mass of acidic organelles that express the late endosome and lysosome glycoprotein LAMP1 (immunoblots,  $0.8 \times 10^6$  cells/track, Fig. 6A). Interestingly, the expression of LAMP-1 was relatively low in PMNLs and significantly higher in lymphocytes, when extracts representing an equal number of cells were immunoblotted.

While the mass of organelles that express LAMP1 may be inferior in PMNLs than in lymphocytes, possibly accounting for a difference of  $V_{\max}$  between the cell populations in quinacrine transport experiments, other explanations must be pursued for the higher affinity uptake in PMNLs. Pinocytosis (fluid phase endocytosis) is sizeable in some phagocytes and facilitates entry of extracellular fluid into the endosomal cell compartment (Pataki, 1995). PMNLs and lymphocytes were compared for their uptake of fluorescent dextran dissolved in the extracellular fluid (37°C, 30 min, Fig. 6B). PMNLs were significantly more intensely labeled than the other cell type (median cell fluorescence intensity of  $44.3 \pm 1.2$  and  $12.7 \pm 1.0$ ,  $P < 0.001$ , Mann-Whitney test). The alternate polymer taken up via pinocytosis, LysoSensor dextran, was also more abundant in granules observed in PMNLs than in those of lymphocytes, but the granules were not more yellowish in any of the cell types (Fig. 6B). This was confirmed by the cytofluorometric analysis: the addition of LysoSensor dextran to cells increased the fluorescence intensity over the autofluorescence by the same ratio of 440 and 535 nm readings for each cell type (data not shown from 2 donors). This supports that, on average, the pH values of freshly isolated PMNLs and lymphocytes do not differ for vesicles accessible to the polymer. Thus, despite a lower mass of LAMP1-positive organelles per cell and a similar pH in the endocytic pathway, PMNLs may capture quinacrine at lower concentrations using a non-concentrating mechanism tentatively identified as pinocytosis working in concert with V-ATPase-mediated concentration.

## Discussion

Quinacrine may be taken as a model of the numerous primary, secondary or tertiary amine drugs from many classes that possess a  $pK_a$  of ~8-10 and are subject to a form of pseudotransport based on ion trapping in acidic cell compartments. Such drugs possess a large apparent volume of distribution, indicative that they may leave the circulation at an accelerated rate. For instance and considering the very small cell volume employed, PMNLs and lymphocytes concentrate quinacrine in the order of 1000-fold from the culture medium in transport experiments reported in Fig. 1. The concentration is even higher if the subcellular distribution of the drug, concerning vacuoles in a fraction only of the cell volume, is considered (Figs 2, 3). Tissue concentration of lipophilic cationic drugs is by itself a source of toxicity and some selectivity for phagocytic cells is suspected (see Introduction). Tissue reservoirs of quinacrine are only slowly released upon cessation of dosing (Ehsanian et al., 2011).

In the present study, we addressed the possible preferential uptake of a model cationic in the major phagocytic leukocyte of human peripheral blood, the short-lived circulating human PMNL, vs. a non-phagocytic leukocyte subtype, the lymphocyte. To do so, a simple quantitative transport model was empirically applied, the hyperbolic function from which  $K_M$  and  $V_{max}$  values were derived, although this is only descriptive for the transporter-free situation that is postulated (pseudotransport driven across membranes by  $\Delta pH$ , see Introduction). The apparent saturability of the transport may derive from the drug buffering of the intravesicular pH. Indeed, in all cell types examined, quinacrine uptake and retention is virtually abolished by the V-ATPase inhibitor bafilomycin A1. However, differences in quinacrine uptake parameters between PMNLs and lymphocytes on one hand, and leukocytes and smooth muscle cells on the other (Marceau et al., 2009; 2013) may be accounted for by modulatory mechanisms specific for cells types, such as differences in intravesicular pH, the presence of membrane transporters (including extrusion pumps) and pinocytosis, a non-concentrating uptake mechanism.

Monocytes do not more actively concentrate quinacrine compared to lymphocytes (Fig. 4), perhaps because the former cell type is an immature form of phagocyte. The most abundant phagocytic cells in peripheral blood, the PMNLs, capture and concentrate most of the quinacrine

applied at low concentrations in a mixture of peripheral blood leukocytes, consistent with a high affinity (low  $K_M$ ) calculated in transport experiments (Fig. 1). Mature macrophages, e.g. those harvested from the lung alveolar space or differentiated *in vitro* from monocytes, were highly competent to concentrate a related drug, amiodarone, in previous studies (Baritussio et al., 2001; Morissette et al., 2009). Quinacrine uptake by cultured human vascular smooth muscle cells or in undifferentiated U937 monoblastoid cells, equally dependent on V-ATPase activity, was comparable in many respects to that of peripheral blood PMNLs, but of lower apparent affinity ( $K_M$  8.7  $\mu$ M and 14.8  $\mu$ M, respectively) and LC3 accumulation occurred at a somewhat higher drug concentration than in PMNLs (present study vs. Marceau et al., 2009; 2013). Cells loaded with amines do not exhibit a detectable nutritional deprivation (Marceau et al., 2012) and the lack of resolution of the vacuolar cytopathology has been likened to Niemann-Pick's congenital lysosomal storage disease (Piccoli et al., 2011), a loss of function of the lysosomal degradation capacity of the cell. Whether this impacts on immunity is unclear at the present time.

While intravesicular pH does not seem to differ between PMNLs and lymphocytes, we have found that the abundance of LAMP1 is much higher in lymphocytes than in PMNLs (Fig. 6A), possibly accounting for a higher  $V_{max}$  of quinacrine transport in the former cell type. This finding is logical if the acidic organelles that express LAMP1 effectively sequester the cationic drug. However, this does not account for the high affinity of the transport in PMNLs and for the selective uptake by these cells at low drug concentrations. **The membrane diffusion rate of quinacrine may intrinsically differ between leukocyte populations. Alternatively,** the higher apparent affinity of quinacrine uptake by the PMNL may be supported by a non-concentrating plasma membrane transport that would collaborate with the intracellular trapping mechanism, thus bypassing the simple diffusion at this level. OCTs, selective for the charged form of cations, are candidates for mediating a plasma membrane transport that does not operate against a concentration gradient (Ciarimboli, 2008). Engler et al. (2011) have observed that peripheral blood PMNLs express a high level of OCT-1 (mRNA, transport of the substrate imatinib suppressed by the inhibitor prazosin) as compared to monocytes and lymphocytes. The cationic antineoplastic drug imatinib is of further interest as a tertiary amine that is known to cause the vacuolar and autophagic cytopathology at micromolar levels (Ertmer et al., 2007; Gupta et al., 2010). However, the lack of effect of decynium-22, an inhibitor of OCT-1 to -3, and of prazosin,

a relatively selective OCT-1 inhibitor, does not support the transport of quinacrine via OCTs in PMNLs. An alternate, non-concentrating transport mechanism is pinocytosis, which delivers droplets of the extracellular fluid to the endosomal apparatus in alveolar macrophages (Pataki et al., 1995). As evaluated using dextran uptake, the PMNLs are the most active cells in this respect in among several tested cell types (including the myeloid cell lines PLB 985 and U937 with  $K_M$  values for quinacrine transport similar to that of lymphocytes, data not shown), with a characteristic subcellular granular distribution similar to that of quinacrine (compare Fig. 6B to Fig. 2).

Whether active extrusion mechanisms modulate quinacrine transport in cells is a legitimate question since P-glycoprotein (the MDR1 gene product) apparently extracts the drug from the mouse brain at the level of vascular endothelial cells (Dohgu et al., 2004). However, freshly isolated human neutrophils reportedly express little P-glycoprotein and its transport function is undetectable; in contrast,  $CD8^+$  lymphocytes and natural killer cells express larger and functional levels (Klimecki et al., 1994).

The neutrophil, representing the vast majority of PMNLs, is rich in various types of granules, and V-ATPase is present in primary (lysosomal) and tertiary granules as well as secretory vesicles (Nanda et al., 1996). Activating PMNLs via the formylated peptide receptors forces the exocytosis of V-ATPase to the cell surface mainly from tertiary granules and secretory vesicles, rendering PMNLs capable of secreting  $H^+$  (Nanda et al., 1996). Pre-stimulation of PMNL with fMLF failed, however, to modify quinacrine uptake in PMNLs (Fig. 1C), suggesting that the bulk of quinacrine uptake is dependent on types of vesicular organelles that are not mobilized by fMLF activation, such as primary granules.

There is a renewed interest in the ability of quinacrine and tertiary amine analogs to act as anti-mitotic drugs in oncology via their binding to DNA and modulation of specific cellular events, such as p53 expression (Gasparian et al., 2012). To observe such an effect, quinacrine concentrations above 5  $\mu M$  are usually necessary, although some analogs may be more potent. We have little morphological evidence for the nuclear uptake of micromolar concentrations of quinacrine in cultured cells based on fluorescence (Marceau et al., 2009; 2012). As for the

functional consequences of quinacrine uptake on leukocytes, previous studies delineate the acute effects of the drug on PMNL functions (Földes-Filep and Filep, 1992) and more chronic actions on T lymphocytes (Namiuchi et al., 1984). Briefly, without exerting any direct effects or cytotoxicity, quinacrine acutely inhibited various responses induced by fMLF (superoxide production, primary granule release, leukotriene B<sub>4</sub> release) with IC<sub>50</sub> values of 230 nM, ~6 μM and 1 μM, respectively, levels that are well above a threshold concentration for vacuolar trapping of the drug (5-20 nM; Figs. 2, 4). Furthermore, quinacrine acted as a weak competitor for [<sup>3</sup>H]fMLF binding to its surface receptors (K<sub>i</sub> 14 μM; Földes-Filep and Filep, 1992). Quinacrine exerted essentially no effect on T cells at 1 μM and some inhibitory ones at 10 μM (phytohemagglutinin- or allogeneic cell-induced DNA synthesis over 3-10 days, T cell-mediated cytotoxicity; Namiuchi et al., 1984). The previous findings may indicate that lymphocytes are generally less sensitive to quinacrine pharmacodynamic effects than neutrophils, in agreement with the transport data generated in the present study. At least the granule release inhibition in PMNLs may be consistent with the general depression of vacuolar traffic seen in cells submitted to massive cationic drug trapping (Marceau et al., 2012).

PMNLs preferentially capture and concentrate the model cationic drug quinacrine, possibly due to the successive effects of a non-concentrating plasma membrane uptake mechanism, tentatively identified as pinocytosis, that collaborates with the classical ion trapping mechanism. Thus, circulating PMNLs represent a site of reservoir formation for cationic drugs that is highly susceptible to macroautophagic cytopathology, with possible **long-term** effects on the immune function (loss of lysosomal function in phagocytes).

### **Conflict of interest**

We have no competing interests for this article.

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## Figure legends

Fig. 1. Uptake of quinacrine into purified peripheral blood leukocyte populations as measured using extract-associated fluorescence. Each experimental point is derived from a 1 ml-tube containing  $10^6$  cells. Values are mean  $\pm$  SEM. of the number of replicates indicate between parentheses.

A, B. Effect of drug concentration on uptake during a 30-min period in PMNLs and lymphocytes, respectively. The average raw data for both cell types (A) were fitted with a hyperbolic function, from which the  $V_{\max}$  values are derived and reported in Table 1. (B) The same drug transport data were normalized as a percent of the transport intensity recorded at the highest tested quinacrine concentration, 30  $\mu$ M, and the corrected  $K_M$  values were derived from these curves and reported in Table 1.

C, D. Time course of quinacrine uptake and effect of bafilomycin A1 (100 nM) on the concentration or retention of quinacrine (C. PMNLs, 2.5  $\mu$ M drug concentration; D. Lymphocytes, 10  $\mu$ M). In C and D, quantities of cell-associated quinacrine observed in the absence or presence of bafilomycin were compared using Student's t test for paired samples (\*  $P < 0.05$ ; \*\*  $P < 0.001$ ).

E, F. Effect of fMLF pre-stimulation (1  $\mu$ M, E) or of co-treatment with decynium-22 (5  $\mu$ M, F, both applied 5 min before quinacrine) on the uptake of quinacrine (various concentrations, 30 min) by purified PMNLs.

Fig. 2. Granular uptake of quinacrine into intact purified PMNLs or lymphocytes and effect of pre- and co-treatment with bafilomycin A1 (100 nM, applied 30 min before quinacrine) (green epifluorescence, 30 min drug uptake). Original magnification 1000  $\times$ .

Fig. 3. Three-dimensional reconstitution from multiple confocal sections (0.4  $\mu$ m-thick) of human PMNLs treated as indicated with quinacrine. Single representative cells are shown. Each cell is viewed from above with an increasing number of planes removed to reveal the intracellular fluorescence distribution, which is essentially granular. The multilobed nuclei are seen in negative. False shadows help visualization. Original magnification, 630  $\times$ .

Fig. 4. Cytofluorometric evaluation of quinacrine uptake by whole peripheral blood leukocytes as a function of the drug concentration (30 min treatment period). A. Cell populations (PMNLs, lymphocytes, monocytes) were identified by their differential side scatter (SSC) and forward scatter (FSC) properties, and their green fluorescence intensity was determined (symbolized by colored dots if superior to the autofluorescence). The fluorescence distribution in the specific leukocyte populations is illustrated in the right hand side columns. Representative results. B. Median green fluorescence intensity as a function of quinacrine concentration in leukocytes subpopulations defined in A. Values are means  $\pm$  SEM of 3 or 4 determinations from 2 donors. **At all quinacrine concentrations, the sets of 3 values corresponding to each** leukocyte subtype was heterogeneous (ANOVA,  $P < 0.001$  in each case). Tukey-Kramer multiple comparison test applied at each quinacrine concentration always indicated that the drug uptake was significantly different in neutrophils vs. either type on mononuclear cells ( $P < 0.01$  at  $1 \mu\text{M}$  quinacrine or  $< 0.001$  at all other concentrations) and that it was never different between monocytes and lymphocytes.

Fig. 5. Processing of endogenous LC3 in purified PMNLs subject to the indicated treatments and evaluated by immunoblotting for LC3 in the total cell extract. A. Effect of time for a  $2.5 \mu\text{M}$  concentration of quinacrine. B. Effect of quinacrine concentration and of bafilomycin A1 on LC3 processing (4-h treatment period). **Bafilomycin A1 was tested alone as a known inhibitor of autophagic resolution (positive control).** Cells ( $1.6 \times 10^6/\text{track}$ ) were maintained in DMEM + 10% FBS for the indicated time period (A) or 4 h (B). To document equal track loading, samples were immunoblotted for  $\beta$ -actin. Replicate number indicated by *n*. In both panels A and B, densitometric values are reported as histograms and were compared with ANOVA ( $P < 0.005$  and  $< 0.05$ , respectively). Dunnett's test was used to compare experimental values with the common control of each experiment, the left-most histogram in both panels (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ).

Fig. 6. PMNLs and lymphocytes compared for the expression of LAMP1 (A,  $0.8 \times 10^6/\text{track}$ , immunoblot representative of 2 or 3 replicates involving 3 different donors for blood leukocytes) and the uptake of fluorophore-labeled dextrans mediated by pinocytosis (B, analysis in Results).

**Figure 1**

**Fig. 1**

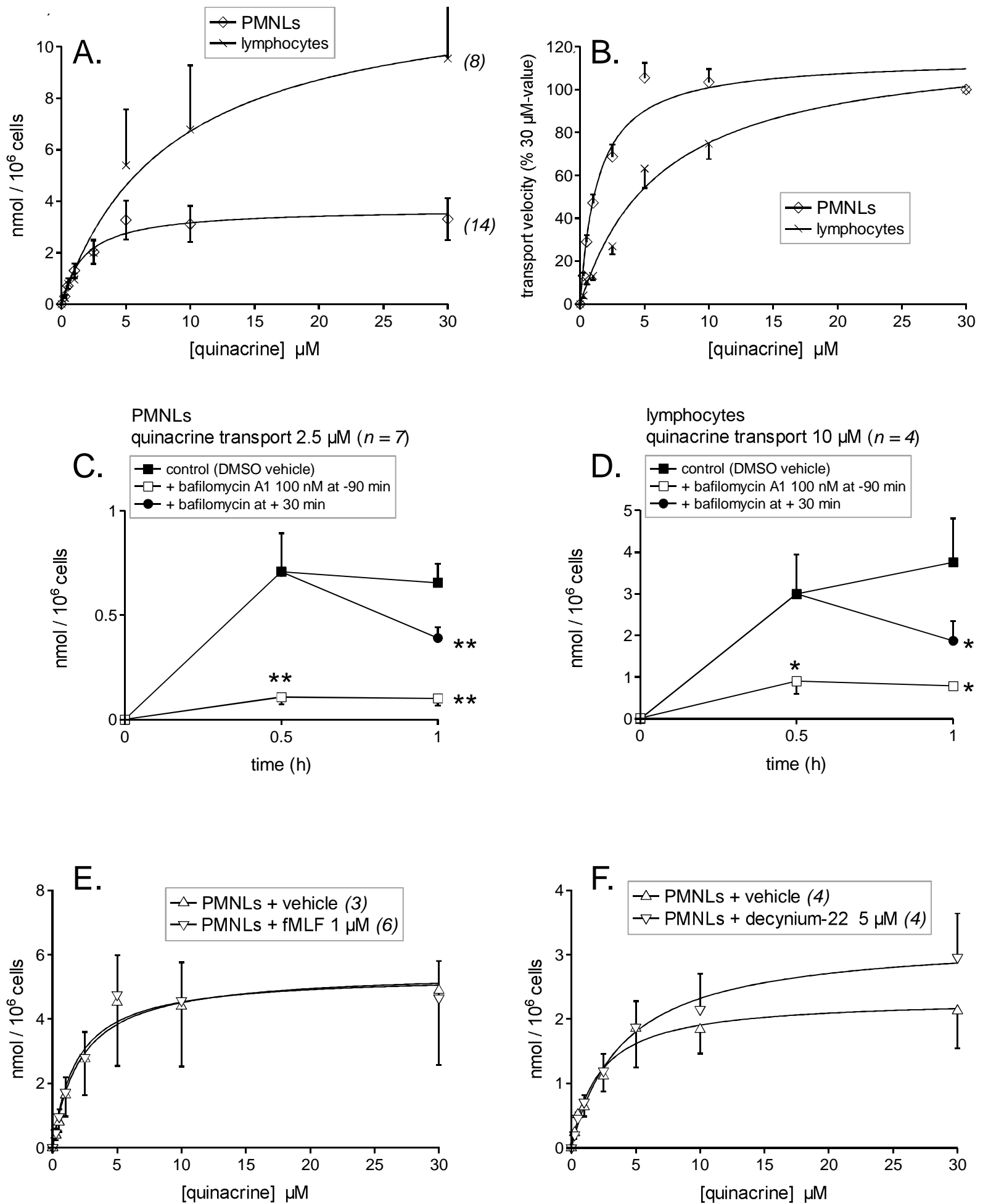


Figure 2

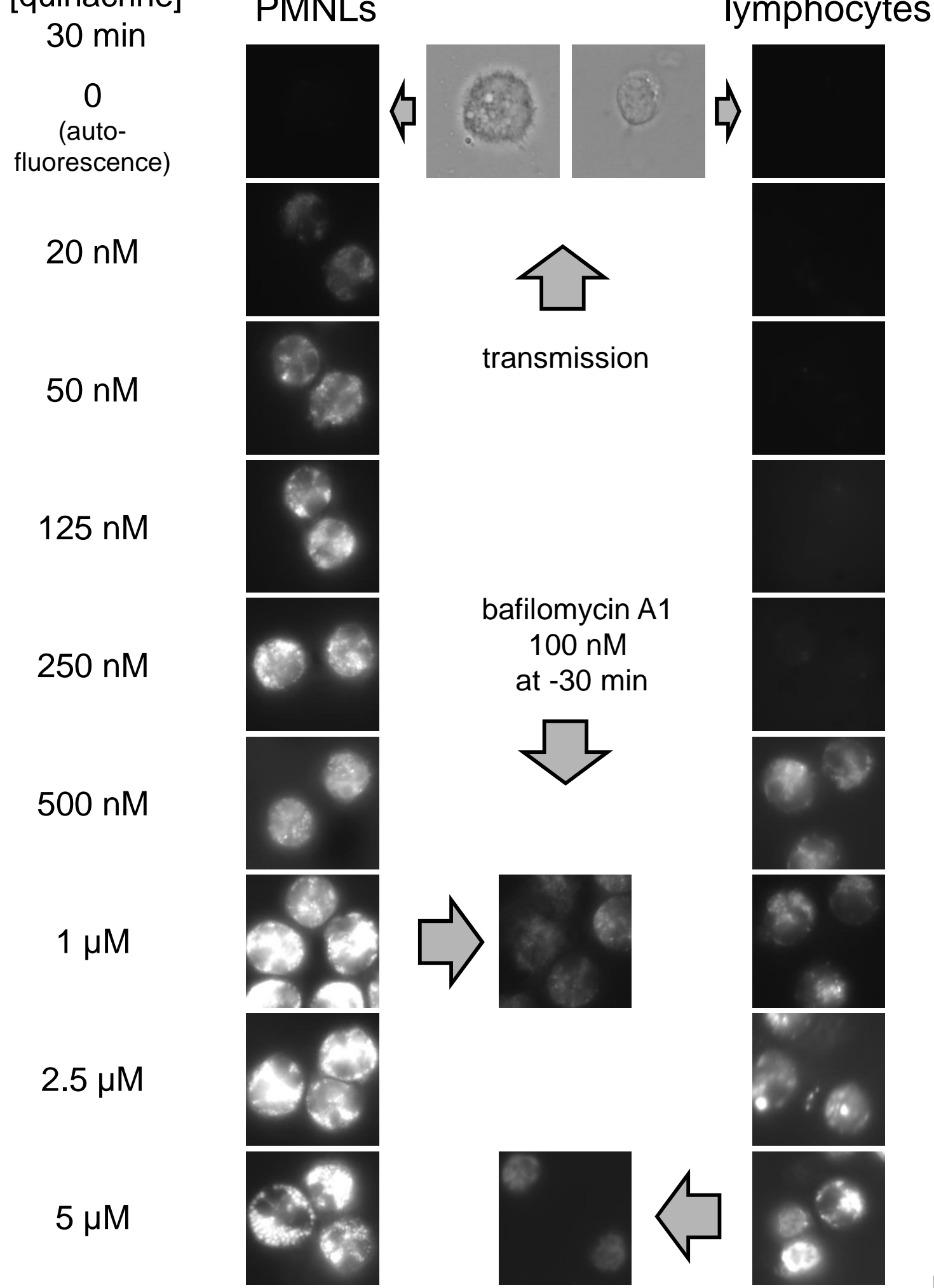


Fig. 2



Figure 3 1  $\mu\text{M}$ , 30 min

5  $\mu\text{M}$ , 30 min

Fig. 3

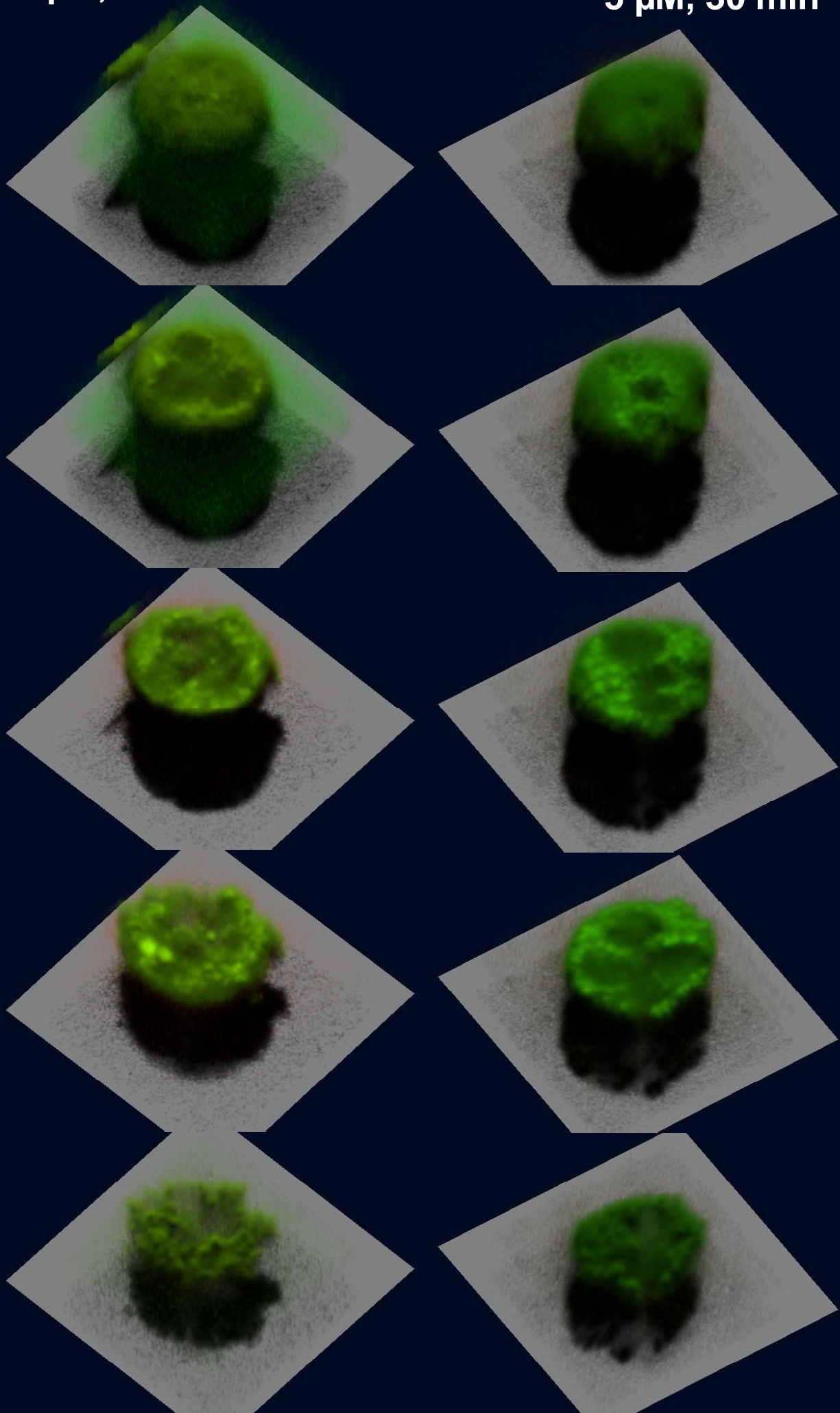
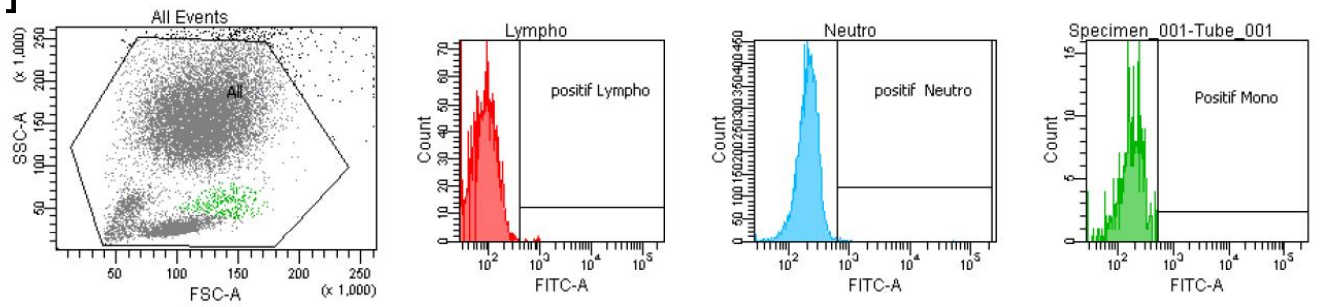


Figure 4

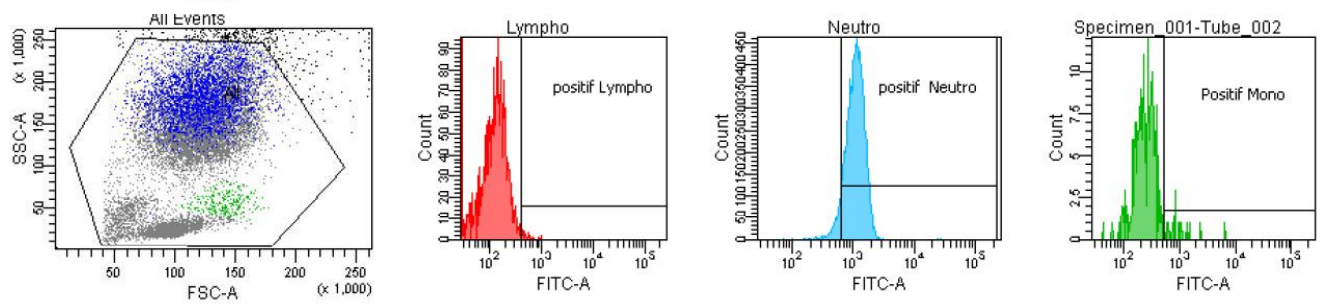
Fig. 4

[quinacrine]

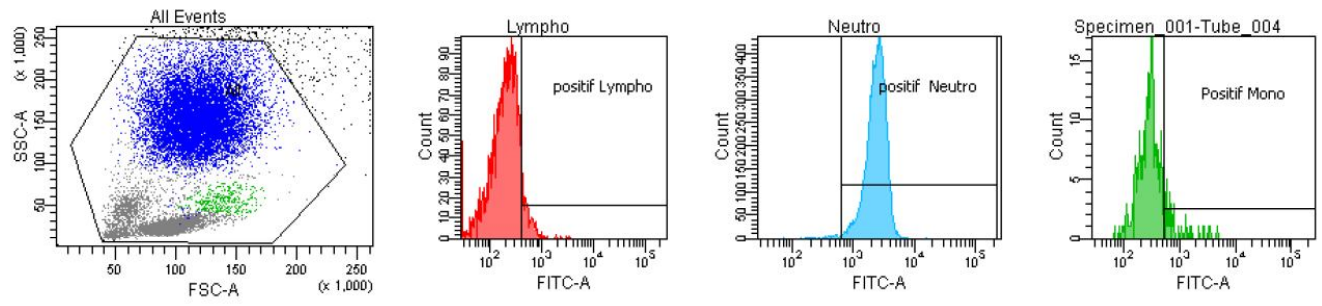
0 (auto-fluorescence)



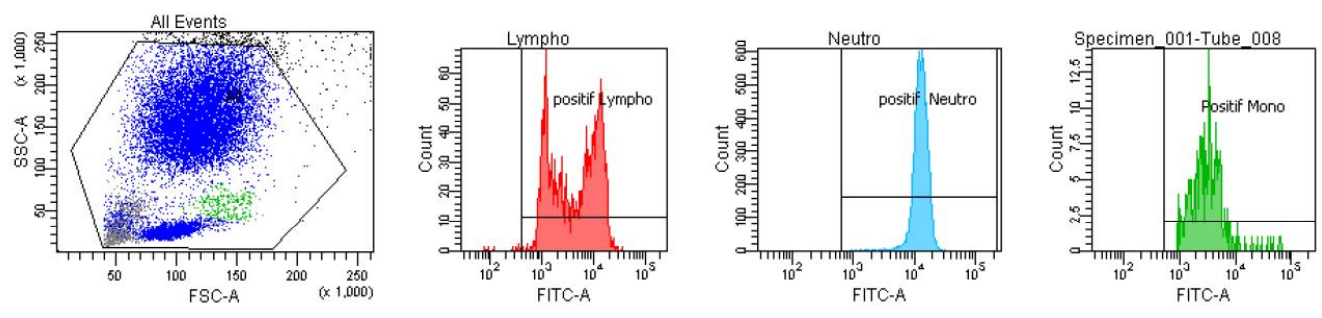
5 nM



20 nM

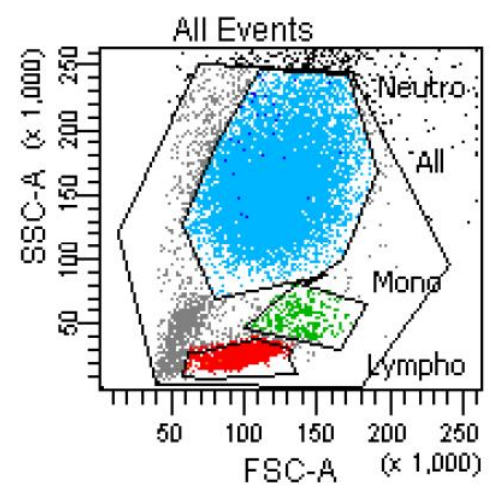
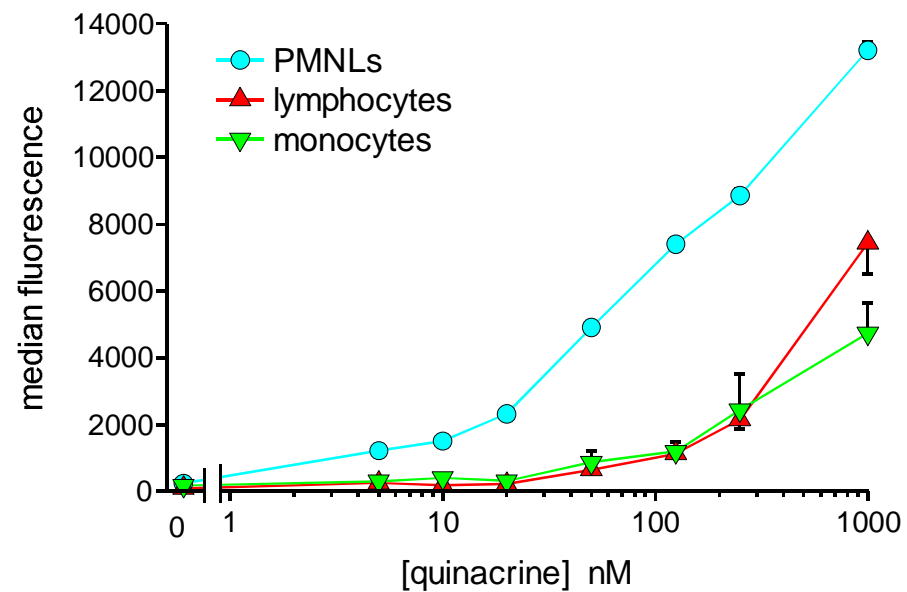


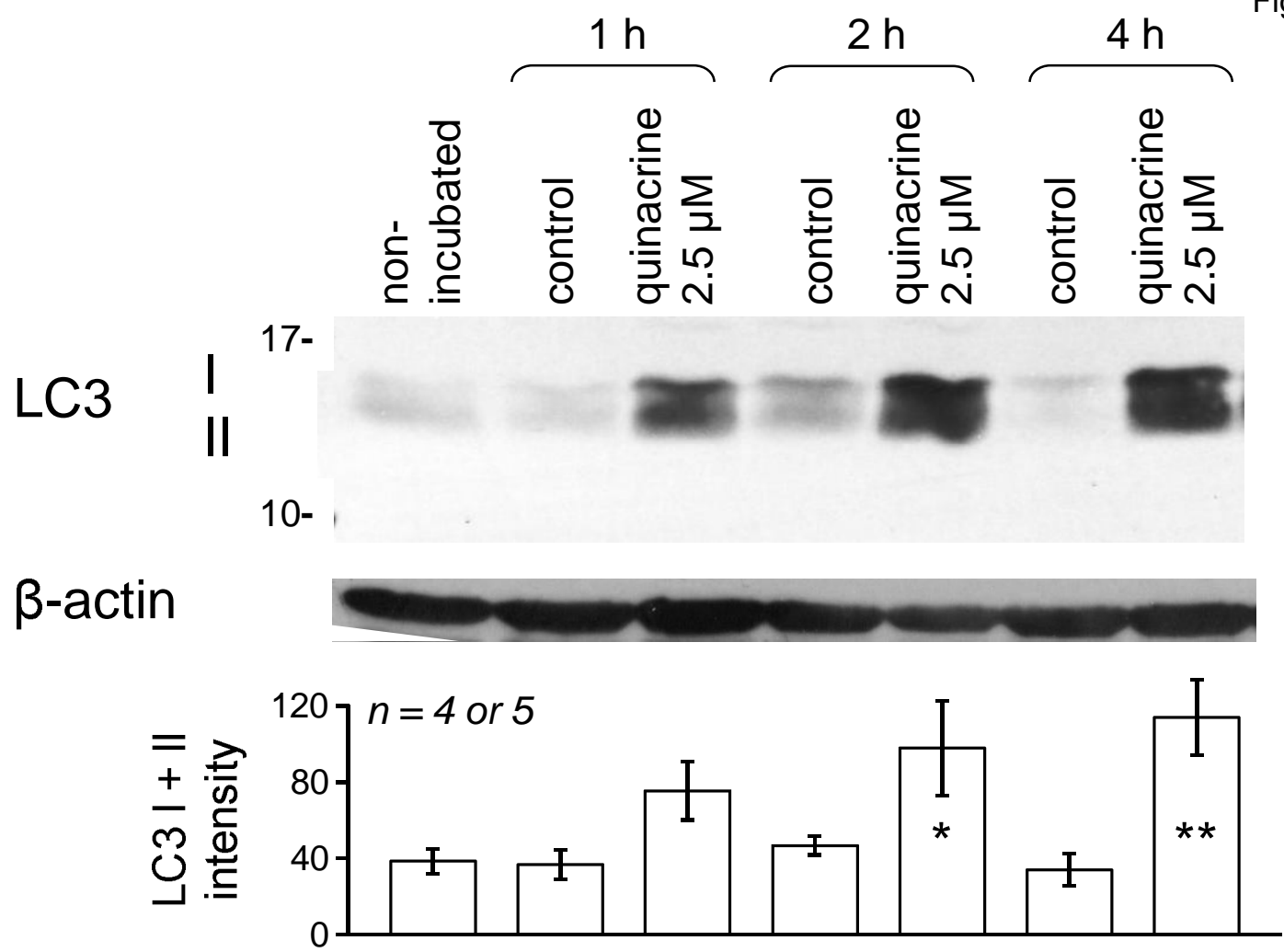
1 μM



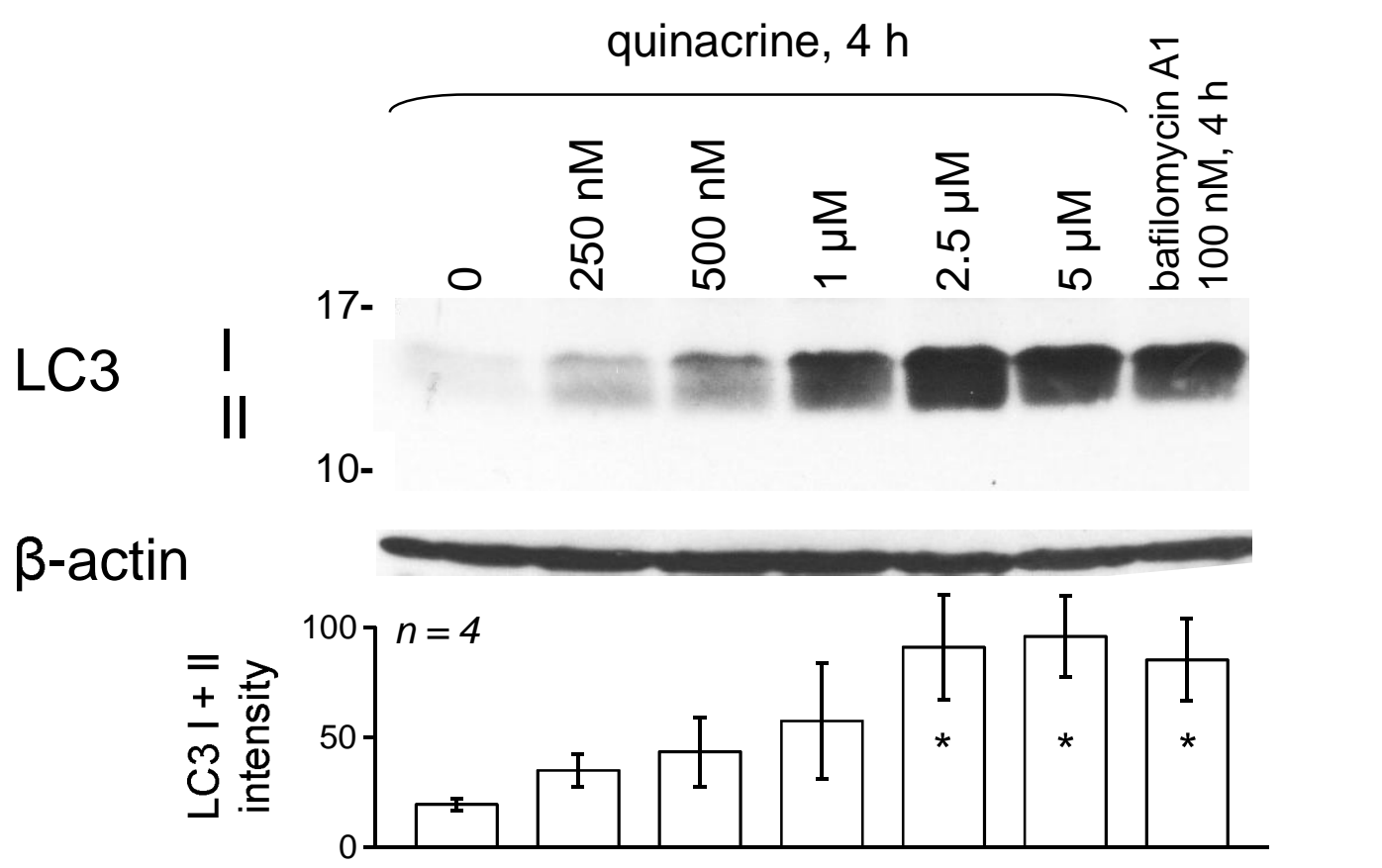
B.

Cytofluorometric analysis, whole leukocytes  
n = 3-4, 2 donors

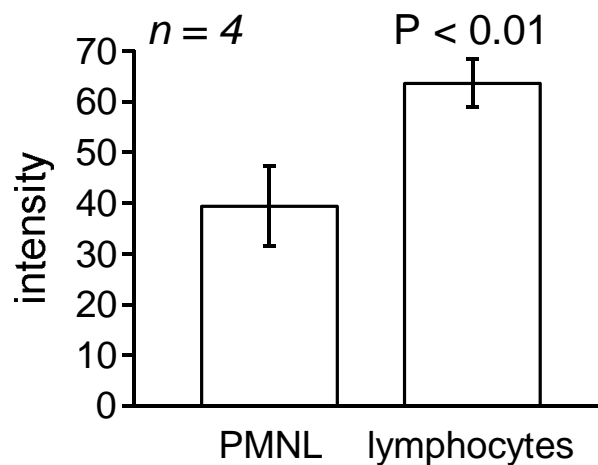
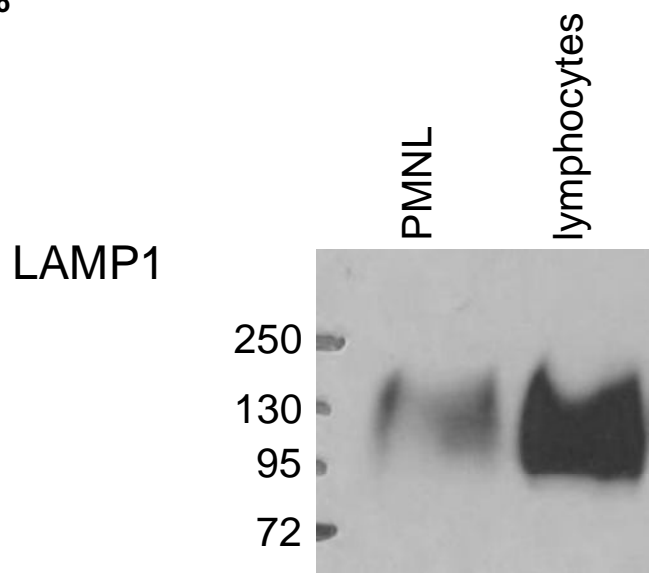




B.



A.



B.

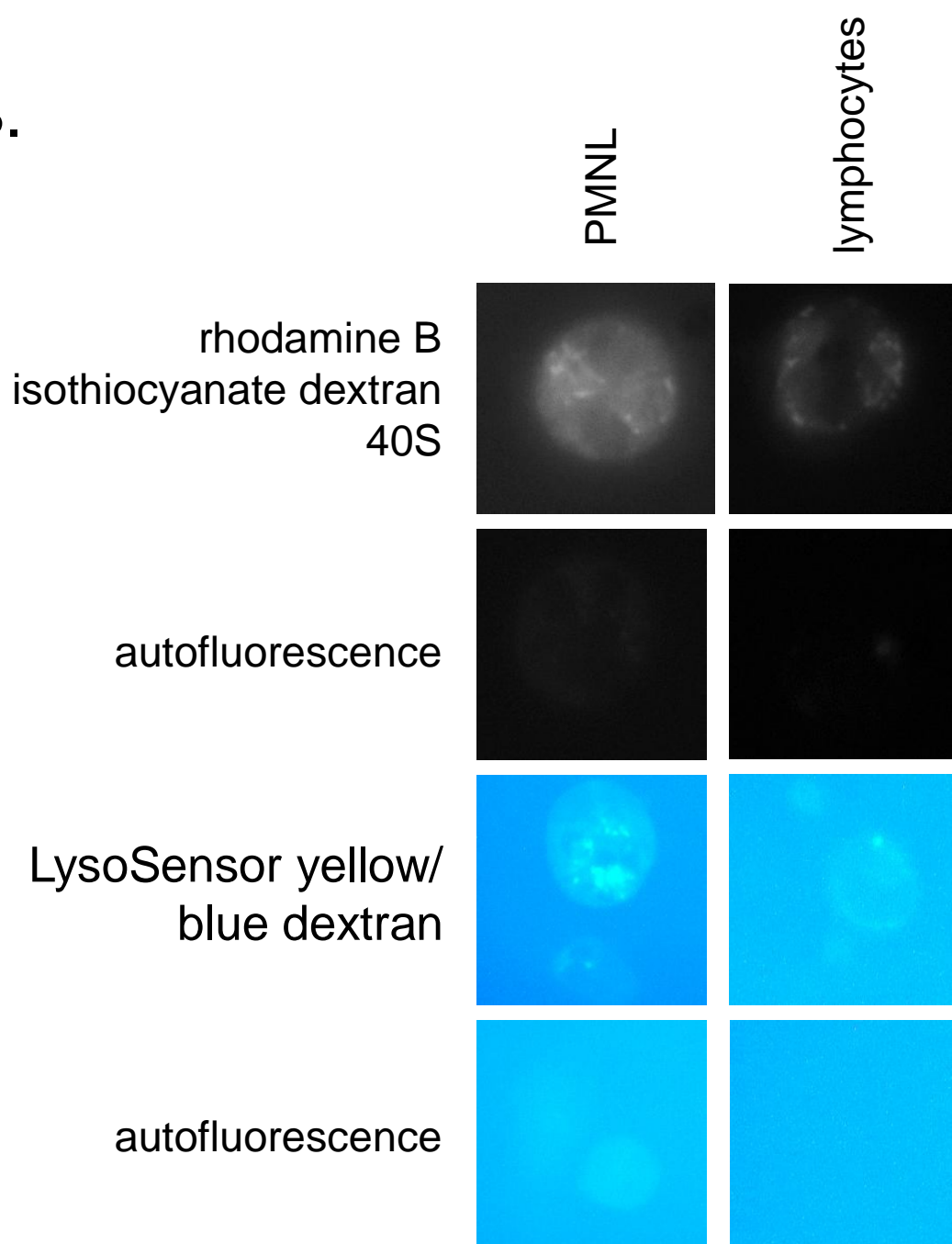


Table 1

Table 1. Quinacrine transport parameters in purified leukocytes.

experimental condition	data from Fig.	corrected $K_M$ ( $\mu\text{M}$ )*		$V_{\text{max}}$ (nmol / 30 min)	
		value $\pm$ SEM	95% confidence limits	value $\pm$ SEM	95% confidence limits
PMNL	1A, B	1.14 $\pm$ 0.17	0.81-1.47	3.71 $\pm$ 0.50	2.71-4.70
lymphocytes	1A, B	6.32 $\pm$ 1.14	4.04-8.60	12.40 $\pm$ 3.72	4.94-19.86
PMNL + vehicle	1E	1.69 $\pm$ 0.37	0.92-2.48	5.48 $\pm$ 1.29	2.78-8.17
PMNL + fMLF	1E	2.01 $\pm$ 0.35	1.30-2.72	5.37 $\pm$ 0.79	3.76-6.97
PMNL + vehicle	1F	2.11 $\pm$ 0.35	1.39-2.83	2.32 $\pm$ 0.34	1.61-3.03
PMNL + decynium-22	1F	3.52 $\pm$ 0.77	1.93-5.11	3.26 $\pm$ 0.48	2.28-4.24

\* corrected  $K_M$  values are derived in all cases from data normalized as a percent of the transport value recorded at the highest tested quinacrine concentration, 30  $\mu\text{M}$ .





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**Declarations**

*Toxicology and Applied Pharmacology* requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

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A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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We have no competing interests for this article.

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**Signature** (a scanned signature is acceptable, but each author must sign)

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