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A novel CCR5 specific pharmacodynamic assay in whole blood using phosphoflow cytometry highlights different ligand dependent responses but similar properties of antagonists in CD8+ and CD4+ T lymphocytes.

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Nonstandard abbreviations used in the paper: PD – Pharmacodynamic, GPCR – G protein-coupled receptor, FSC – Forward scatter, SSC – Side scatter, FACS - Fluorescence-activated cell sorting

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Abstract

CCR5 is a major drug target for both inflammation and virology indications. The primary function of CCR5 is to mediate the trafficking of CCR5 expressing lymphocytes to any of the CCR5 ligands, which are often increased during inflammatory responses. Additionally, CCR5 is a co-receptor for HIV, mediating R5 tropic HIV infection of CCR5 expressing CD4 T cells. We report the use of a novel method to assay the pharmacodynamic (PD) properties of small molecule and antibody inhibitors of CCR5 ligand induced activation by measuring phosphorylation of serine residue 349 (S349) in the cytoplasmic tail of human CCR5 using phosphoflow cytometry in whole blood. This assay is highly specific and measures CCR5 phosphorylation in both CD8+ and CD4+ T cells and allows the calculation of inhibitor IC50 values from both lymphocyte subsets in the presence of CCR5 antagonists. Additionally, this assay is cross reactive to non-human primates and allows PD analysis in whole blood from rhesus and cynomolgus macaque. Using this assay we identified different ligand dependent response properties between CD8+ and CD4+ T cells although CCR5 antagonists behave with similar properties against both cell types. The use of this assay may be of particular benefit to monitor PD effects of CCR5 inhibitors during drug development, preclinical *in-vivo* studies, and in patients currently being treated for HIV or CCR5 mediated inflammatory diseases with CCR5 inhibitors. Similar phosphoflow approaches to other GPCR targets on circulating lymphocytes may prove to be the most reliable PD assay for pre-clinical and potentially clinical development.

Introduction

CCR5 (Chemokine-CC-motif-receptor 5) is a 7 transmembrane G-protein coupled receptor (GPCR) classified as a chemokine receptor (IUIS/WHO Subcommittee on Chemokine Nomenclature, 2003). CCR5 mediates the trafficking of CCR5 expressing lymphocytes to the CCR5 ligands, MIP-1α (CCL3), MIP-1β (CCL4), Rantes (CCL5), or MCP-2 (CCL8) (Samson et al., 1996; Charo and Ransohoff, 2006). It is expressed primarily on subsets of CD8+ T cells, CD4+ T cells and monocytes/macrophages (Geginat et al., 2001; Tomkowicz et al., 2006). Current methods to assay PD activity of CCR5 inhibitors include chemotaxis, intracellular calcium responses, MAP kinase activity and viral infection. All of these assays have limitations in that they require either transformed cells, artificial cell lines with over expression of CCR5 and/or long incubation times to observe functional outcomes, many of which introduce an element of artificiality into the experimental equation. Another method used to validate CCR5 inhibitors is to monitor the presence or lack of CCR5 surface expression in the presence of ligand assuming that this corresponds to internalization of the receptor. Although this assay format appears to supply useful data, it does not rule out the possibility that CCR5 inhibitors are impairing detection of all CCR5 molecules, especially in situations where large molecule inhibitors such as antibodies are being evaluated. The current method was developed to observe the most immediate effects of CCR5 activation in normal CD4+ and CD8+ T lymphocytes in human whole blood with the intention of extending studies into non-human primates for the evaluation of clinical drug candidates.

GPCR share a common system of context dependent activation, regulating their responsiveness to varying ligand concentrations. Desensitization occurs via multiple mechanisms such as GPCR mediated activation of GPCR kinases (GRKs), protein kinase A, protein kinase C, and mitogen activated protein (MAP) kinases, resulting in receptor phosphorylation at sites on the intracellular loops and carboxyl-terminal tail (Gainetdinov et al., 2004). After phosphorylation the receptor becomes a binding substrate of arrestin proteins, which prevent the receptor from activating additional G proteins. The GPCR phosphorylation mechanism also facilitates other functions, such as receptor internalization from the cell surface through clathrin-coated pits, and allowing signaling molecules to initiate G protein-independent pathways such as second messenger activated pathways which contribute to a cell's responsiveness to agonist. GPCR desensitization and subsequent signaling pathways have been reviewed extensively (Lefkowitz and Shenoy, 2005; Premont and Gainetdinov, 2007).

In the case of CCR5, after ligand induced activation, multiple serine residues in the cytoplasmic C-terminus of CCR5 are phosphorylated, likely by the GRK family of protein tyrosine kinases (Pitcher et al., 1998; Pollok-Kopp et al., 2003). These phospho-residues allow binding of beta arrestin, which mediates receptor internalization (Bohm et al., 1997). Phosphorylation of one of these sites, serine 349 (S349 CCR5, nomenclature corresponding to the human CCR5 sequence) is the primary readout of this assay. Using a monoclonal antibody, clone E11/19 (Pollok-Kopp et al., 2003), we have developed a method of reliably detecting the phosphorylated form of S349 CCR5 using phosphoflow cytometry in whole blood samples.

Phosphoflow cytometry is a method to detect phosphorylated proteins in single cells using a phospho specific antibody measured by a flow cytometer and has been demonstrated in the setting of drug discovery (Krutzik et al., 2004; Perez OD, 2004; Krutzik et al., 2008). This differs from traditional flow cytometry used to detect and quantify the presence of surface or intracellular molecules such as cytokines relative to control cell populations. Phosphoflow detects activation induced changes of signaling molecules inside the cell relative to unstimulated populations of identical cells which can be in the same sample.

We have used this assay to characterize the concentration dependent response of CD4+ and CD8+ T cells to human MIP-1β. CCR5 is expressed higher on CD8+ T cells than CD4+ T cells in both human and non-human primate species (Desmetz et al., 2006; Pandrea et al., 2007). Moreover, the higher expression on CD8+ T cells results in higher receptor surface density and enhanced chemotactic responses (Desmetz et al., 2006). We observed a corresponding similarity in the pS349 CCR5 response to MIP-1β stimulation. The ligand induced pS349 CCR5 response was completely inhibited using CCR5 small molecule inhibitors Schering X (Vierboom et al., 2005), Maraviroc (Celsentri) (Dorr et al., 2005), or a novel anti-CCR5 monoclonal antibody. Moreover, the specificity and sensitivity of this assay was used to determine IC50 values of CCR5 inhibitors directly in whole blood. We observed that the responses of CD8+ and CD4+ T cells to MIP-1β were different although the PD properties of antagonists against each cell type were similar.

Methods

Whole blood – Freshly drawn, normal human whole blood in sodium heparin was obtained from voluntary blood donors by Roche Palo Alto Occupational Health Services. Freshly drawn normal cynomolgus macaque whole blood in sodium heparin was obtained from Roche Palo Alto. Freshly drawn normal rhesus macaque whole blood in sodium heparin was obtained from the California National Primate Research Center.

Stimulation, Phosphoflow and FACS staining – Whole blood was incubated at 37ºC with or without specified concentrations of small molecule or antibody inhibitors for either 30 minutes (small molecules) or 1 hour (antibodies) and then stimulated with PBS or recombinant human MIP-1β (R&D Systems) for an additional 30 minutes at 37ºC. Whole blood was then treated with Lyse/Fix buffer (BD Biosciences) for 10 minutes as described by the manufacturer. Whole blood cells were then washed once in staining buffer (PBS + 10% BSA), resuspended in 100% ice cold methanol, and vortexed vigorously. Samples were then stored at -80ºC and stained for FACS analysis within 18-72 hours. Antibodies for FACS analysis were obtained as follows: Anti-pS349 CCR5 PE (clone E11/19) and anti-CD8α Alexa 647 (clone RPA-T8) were from Biolegend. Anti-CD4 PerCP-CY5.5 (clone L200) was from BD Biosciences. Normal mouse serum from Rockland was used to block non-specific antibody binding prior to staining. For experiments in which we performed surface staining, human whole blood was treated with BD Pharm Lyse buffer (BD Biosciences) as described by the manufacturer. Following washes, cells were blocked with anti-human CD32 (clone FL18.26, BD Biosciences) and stained with mouse anti-CCR5 (clone 3A9, BD Biosciences). FACS

data was acquired on a FACS Caliber flow cytometer (BD Biosciences) using Cell Quest software. Between 500,000 and 1,250,000 total events were acquired on average.

Analysis of Phosphoflow

FACS data was analyzed using Flowjo software (Tree Star Inc.). A tight lymphocyte gate was identified in the FSC vs. SSC plot of all events acquired. From that gate, CD4+ and CD8+ events were gated. From each of these gates a plot was made to analyze the staining pattern of pS349 CCR5 PE vs. an unstained FL-1 parameter. In some assays there was fluctuation from sample to sample in the pS349 CCR5 PE staining intensity, particularly in experiments where many samples were processed. To adjust for changes in the general position of the gate, the Schering X treated control sample was used to create a gate around the mass of the events which were non-responsive. Then, this gate was applied to every sample and its position was adjusted to match the minor fluctuations in the position of the non-responsive cells. Events that were acquired above this gate were considered pS349 CCR5 PE+. In rhesus and cynomolgus an additional CD4+ CD8+ double positive lymphocyte population was observed and analysis of the pS349 CCR5 response in the gate demonstrated data similar to that observed in the CD4+ T cell gate (data not shown).

Small molecule and antibody inhibitors of CCR5 – The CCR5 small molecule inhibitors Schering X (SchX) and Maraviroc (aka Celsentry, Selzentry) were produced at Roche Palo Alto (Vierboom et al., 2005; Dorr et al., 2005). The anti-CCR5 monoclonal antibody, CCR5-MAB1, was from Roche Palo Alto.

Statistical analysis

Statistical significance was determined with the two-tailed, unpaired Student's t-test.

Results

Stimulation of human whole blood with 100 ng/ml MIP-1β for 30 minutes lead to an increase in the percentage of pS349 CCR5+ cells in both the CD8+ and CD4+ T cell gates. This response was completely inhibited by preincubation for 30 minutes with the specific CCR5 inhibitor Schering X. The potency of Schering X was confirmed by determining the IC_{50} value in cell based chemotaxis assays. We chose to use it at 10 uM for all future experiments as a positive control in which the activity of ligand induced CCR5 activity was completely inhibited (Figure 1a). We consistently observed a level of background activation prior to MIP-1β induced activation, which varies from donor to donor. This is likely due to low levels of endogenous CCR5 ligands in circulation at the time of the blood draw and may correlate with the level of inflammation in the donor. This must be considered when thinking about the actual concentration of stimulation, since stimulation will be the sum of endogenous CCR5 ligands and the exogenously added MIP-1β and may be a source of variability from donor to donor. Similar data was observed using other CCR5 ligands such as Rantes (data not shown). We performed this assay in over 30 individuals to understand how much variability there was in the response to MIP-1β. There was significant quantitative variability in the response from donor to donor. However, every single donor demonstrated a qualitative response to exogenously added MIP-1β, above the background level, in both CD4+ and CD8+ T cells, and this response was completely inhibited by Schering X (Figure 1b). To determine how the pS349 CCR5 response correlated with CCR5 surface expression, whole blood from 9 human donors was stimulated with MIP-1β and the pS349 response was assayed while surface expression of CCR5 was simultaneously compared using a separate staining

procedure. Although there was considerable variability in the surface expression of CCR5 among the donors in both CD8+ T cells (30.91% +/- 4.69% SEM) and CD4+ T cells (9.88% +/- 0.87% SEM), the pS349 response was consistently less than the level of CCR5 surface expression in both CD8+ T cells (15.35% +/- 2.07% SEM) and CD4+ T cells (3.04% +/- 0.57% SEM), which further supports the specificity of this assay (Figure 1 c). We defined the maximum possible pS349 response to be equal to the CCR5 surface expression. Thus, using a stimulus of 100 ng/ml MIP-1β for 30 minutes, the response in CD8+ T cells was 55.05% +/- 7.027% SEM and the response in CD4+ T cells was 30.97% +/- 4.073% SEM (Figure 1 c). CD8+ T cells demonstrated a more robust quantitative response than the CD4+ T cells suggesting that the response to CCR5 varies in a cell type specific manner within the same whole blood sample.

To determine the appropriate duration of stimulation to test the efficacy of CCR5 inhibitors we stimulated the whole blood from human donors with 100 ng/ml MIP-1β and immediately fixed the cells at 4, 10, 15, 30 and 60 minutes. We observed a clear time dependent activation with some activation observed as soon as 4 minutes and maximal activation observed between 30 and 60 minutes in both CD8+ and CD4+ T cells. Interestingly, the level of activation was not reduced by even 60 minutes, demonstrating a very stable window during which to perform our assays (Figure 2). Based on this data we decided to use a 30 minute stimulation time in subsequent assays.

To determine if this assay could be used to analyze a concentration dependent activation response, we stimulated the whole blood from human donors with increasing

concentrations of MIP-1β for 30 minutes. A concentration dependent response was observed. As the concentration of stimulus increased a subset of responding cells was observed to separate from the mean fluorescence of the total cell population and at high levels of stimulation this separates into a distinct population (Figure 3a). From these FACS plots, numerical data was plotted to analyze the concentration response curve (Figure 3b). Again, we observed variability in the response from different donors in CD8+ and CD4+ T cells. In the CD8+ T cell gate, some donors reach a plateau by 900 ng/ml MIP-1β whereas others do not. One example of each donor is shown. In the CD4 gate, none of our donors reached a plateau in the stimulation, even at 900 ng/ml MIP-1β. To analyze the PD effects of specific CCR5 inhibitors we sought to use a stimulation that would give a clear but sub-optimal response in most donors. We therefore chose to use a concentration of 100 ng/ml MIP-1β in subsequent experiments.

To determine the PD effects of Maraviroc in this system, a CCR5 specific antagonist currently in the clinic, we pretreated human whole blood with increasing concentrations of Maraviroc and then stimulated the cells with 100 ng/ml MIP-1β (Dorr et al., 2005; Este and Telenti). We observed a MIP-1β dependent induction of pS349 in CD8+ and CD4+ T cells and this response was inhibited by Maraviroc in a concentration dependent manner (Figure 4a). This data was used to determine IC50 values (Figure 4b). Interestingly, we observed similar IC50 values for Maraviroc in CD8+ T cells (0.57 nM) and CD4+ T cells (0.44 nM) indicating that the efficacy of a specific concentration of a CCR5 inhibitor is not necessarily cell type specific, at least when considering CD8+ and CD4+ T cells. This similar PD effect was consistently observed with every small

molecule CCR5 inhibitor that we tested (Roche compounds in development or otherwise). Although IC50 values were identified for both T cell types, we consider the data from the CD8+ T cells to be the most representative of the properties of specific CCR5 inhibitors due to consistently higher levels of CCR5 expression and consistently higher induction of pS349 by CCR5 ligands compared to CD4+ T cells.

In addition to small molecule inhibitors of CCR5, we have developed neutralizing antibodies against CCR5. We used this assay to study the PD effects of CCR5-MAB1, an anti-CCR5 monoclonal antibody that was generated by Roche. Preincubation of human whole blood with CCR5-MAB1 inhibited the MIP-1β induced pS349 response in a concentration dependent manner (Figure 5a). We observed similar IC50 values for CD8+ T cells (0.067 nM) and CD4+ T cells (0.080 nM) (Figure 5b). The potency of CCR5-MAB1 was superior to that of small molecule CCR5 inhibitors.

This assay was designed to cross react with non-human primate species, therefore antibodies known to cross to rhesus and cynomolgus macaque were chosen. However, the specificity of the anti-human pS349 CCR5 antibody had not been determined. We tested whole blood from rhesus and cynomolgus stimulated with human MIP-1β. In both rhesus and cynomolgus, human MIP-1β stimulated a response that was detectable by the anti-human pS349 CCR5 antibody, similar to that observed in human whole blood (Figure 6a). The response was completely inhibited by Schering X. A similar response was observed when cynomolgus whole blood was stimulated with recombinant cynomolgus MIP-1β (data not shown). Furthermore, we tested Maraviroc in cynomolgus

whole blood and found that it inhibited the human MIP-1β induced phospho-CCR5 response in a concentration dependent manner similar to that observed in humans (Figure 6b).

Discussion

We report a reliable method to assay the PD effects of CCR5 inhibitors in whole blood using phosphoflow cytometry. Phosphoflow cytometry, is different from traditional flow cytometry used to detect and quantify surface molecules or intracellular molecules such as cytokines relative to isotype control treated cells. Phosphoflow on the other hand detects activation induced changes of signaling molecules inside the cell relative to control unstimulated populations of identical cells. In this assay, we induce the activation of CCR5 expressing cells for a determined duration of time, then immediately fix the cells, followed by permeablization of the cells to allow the detection antibody to access signaling molecules inside the cell. A quantitative increase in phosphorylated S349 CCR5 is observed in a time and concentration dependent manner, and this response is inhibited by the prior addition of CCR5 specific antagonists before ligand stimulation in whole blood. This readout can be combined with antibodies which identify cell specific markers, such as CD4 and CD8 on T cells, and used to identify the PD effects in discrete lymphocyte populations known to express CCR5 on their surface.

This assay has benefits over other conventionally used assays to measure inhibition of GPCR activity. The main benefit is that it measures the activity of small molecule or antibody inhibition simultaneously in multiple natural target cell populations in whole blood. Another benefit is that it measures one of the most proximal signaling events occurring immediately downstream of ligand binding. These benefits give a more clear view of the effects of compounds by reducing the artificiality introduced into experimental systems through the use of transfected and/or immortalized cell lines,

inducible receptor expression systems, extensive manipulation to purify primary cell populations, and long durations of culture necessary in assays such as chemotaxis. Other applications include the ability to test compounds in blood from multiple individuals to observe the variability in response, as well as testing blood from various non human primate species. These applications significantly increase the confidence in the properties observed during testing of the compounds. This does not however represent a complete analysis of the effects of compounds and we recommend that this assay be used in combination with other assays such as those previously described to get a more complete representation of the PD effects of an antagonist.

CCR5 in human whole blood has been shown to be expressed on CD8+ and CD4+ T cells (primarily of the effector memory subsets of each), as well as a subset of monocyte/macrophages (Geginat et al., 2001) (Tomiyama et al., 2002; Takata and Takiguchi, 2006; Tomkowicz et al., 2006). Ideally, one would like to look at the stimulated response specifically in CCR5 expressing cells. However, phosphoflow requires the cells to be fixed and then permeablized by methanol and this can have detrimental effects upon antibody recognition of target epitopes (Krutzik et al., 2005). We found this treatment of the cells resulted in undetectable staining by all currently available anti-CCR5 antibodies, likely due to changes in binding epitopes on CCR5. Thus we were unable to incorporate a CCR5 specific gating strategy into our analysis. In preliminary experiments we observed multiple T cell subsets which may variably express CCR5, particularly in cynomolgus monkeys, and especially in individuals or animals with ongoing inflammation. Indeed, in humans multiple effector populations of CD8+ T

cells have been identified in peripheral blood . In an effort to keep this assay reproducible, simple to run in a clinical laboratory, and simple to analyze, we decided to focus our analysis on total CD8+ and CD4+ T cells in the lymphocyte gate.

In this study we used MIP-1β because it is the most specific CCR5 ligand, unlike other CCR5 ligands such as RANTES, MIP-1α or MCP-2 that also act as agonists of other receptors such as CCR1, CCR2 and CCR3. However, there are reports that MIP-1β may signal through CCR1 at high concentrations and it is conceivable that signaling molecules downstream of CCR1 could phosphorylate CCR5 and lead to cross-desensitization. Heterodimerization involving a CCR1/CCR5 complex could theoretically lead to cross-desensitization. Although we have not formally explored the possibility of desensitization via any of those mechanisms, it seems unlikely that such process is at play as we tested multiple CCR5 small molecule and antibody antagonists that were cross screened against CCR1 to confirm CCR5 specificity. In the presence of MIP-1β stimulation, we consistently observed complete inhibition of the pS349 CCR5 response, suggesting that even if MIP-1β were signaling through CCR1, the signal does not induce phosphorylation of S349 CCR5, although we can not rule out phosphorylation of other CCR5 residues.

A majority of the CCR5 signal transduction investigations have been done using monocyte cell lines which describe an ERK phosphorylation response detectable by western blotting or other methods (Tomkowicz et al., 2006). We included an anti-ERK1/2 antibody in our preliminary experiments in human whole blood and surprisingly

we did not observe any ERK1/2 activation in CD8+ or CD4+ T cells, even though theses cells demonstrated clear MIP-1β induced pS349 CCR5 responses (data not shown). Alternatively, when we moved our gating criteria away from the tight lymphocyte gate and into other distinct cell populations in the FSC vs SSC data plot some of which may represent monocytes populations, we observed a rapid and transient MIP-1β induced phospho ERK1/2 response in a subset of cells which did not demonstrate any detectable pS349 CCR5 response. It is likely that these cells were a subset of monocytes based on their size and granularity, although extensive characterization was not performed. This suggests that although CCR5 ligands may induce signal transduction events downstream of CCR5, specific events are cell type specific. It is likely that T cells do not express the signaling molecules (or they are otherwise unavailable) necessary to couple an ERK1/2 response to CCR5 while the putative monocyte cell subset does have these molecules but may not express the kinase responsible for S349 phosphorylation. Data such as this helps to explain some of the pleotropic effects of cytokines and chemokines in diverse cell types and emphasizes the strength of phosphoflow in its ability to analyze these pathways simultaneously in multiple cell types from a single sample.

During the course of running this assay we observed that during the winter and spring months our response in CD4+ T cells was higher than that observed in the summer and fall months, and the opposite was observed for CD8+ T cells. Although we did not test enough individuals to demonstrate statistical significance across time, we speculate that there is a seasonal rhythm in the qualitative response of CD8+ and CD4+ T cells. Previous studies have identified seasonal variability in the number of cells circulating in

whole blood with each cell type having a distinct harmonic (Maes M, 1994). That is, CD4+ T cells, CD8+ T cells, monocytes, neutrophils, B cells, etc. demonstrated unique rhythms and amplitudes of change. Although unreported, we speculate that in addition to seasonal variability in cell number, there may be a seasonal effect on the responsiveness of cells to specific stimuli such as the one induced by MIP-1β through CCR5 on CD4+ and CD8+ T cells. This may be regulated, at least in part, by alterations in the level of CCR5 expression, although a previous report has described the density of CCR5 expression on CD4+ T cells as relatively stable over short time periods of less than four months (Jacques Reynes, 2000). Alternatively, the levels of down stream signaling molecules or relative sensitivity due to the temporal proximity of activation may enhance responsiveness. If the response fluctuates due to alterations in CCR5 expression on CD4+ T cells, which functions as a co-receptor for HIV, there should be seasonal correlation with HIV infection rates. Indeed, CCR5 cell density correlates with HIV RNA plasma level and with R5 HIV infectivity (Jacques Reynes, 2000; Heredia, 2007). Although time of infection can not been documented for most cases, newly diagnosed HIV infections were higher in the fall-winter months (Kathleen A. Brady MD, 2005). A more extensive study regarding the seasonal variability in CCR5 expression, as well as the seasonal CCR5 responsiveness to ligand induced activation should illuminate these issues, especially if it will affect data from clinical trials performed in multiple centers with different seasonal components. This may allow identification of specific seasons that would be best to treat patients with CCR5 inhibitors for anti-inflammatory purposes or anti-viral CCR5 inhibitors for HIV.

The use of any phosphoflow assay depends on a good antibody to measure specific amino acid phosphorylation following stimulation. For most GPCR, no phospho-receptor specific antibodies have been identified yet although there are potentially multiple phosphorylated targets in the signaling cascades of many receptors in whole blood that may generate robust read outs using other phospho-specific antibodies. Based on our experience evaluating CCR5 antagonists, the strength of the data generated using phosphoflow and the use of the most relevant population of target cells in whole blood strongly suggests that similar phosphoflow assays will have considerable strength when applied against other GPCR targets on circulating lymphocytes.

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Legends for Figures

Figure 1. Detection of phosphorylated S349 of CCR5 in human whole blood. Human whole blood was stimulated with 100 ng/ml MIP-1β for 30 minutes and analyzed using phosphoflow cytometry. Gating tightly on lymphocytes and then on either CD8+ or CD4 + events, the response to MIP-1β was measured by the detection of pS349 CCR5. Representative data from 2 donors is shown (a). The assay was repeated using multiple donors and the cumulative data compared (N=31) using the student's T test (b). Surface expression of CCR5 was compared to the pS349 CCR5 response in 9 human donors using the paired student's T test (c).

Figure 2. Time course of S349 CCR5 phosphorylation demonstrates a stable and sustained response. Human whole blood was stimulated with 100 ng/ml MIP-1β for the indicated time and the pS349 CCR5 response was detected in both CD8+ and CD4+ T cells. Representative data from three donors is shown.

Figure 3. Concentration dependent induction of pS349 CCR5 response. Human whole blood was stimulated with increasing concentrations of MIP-1β for 30 minutes and the pS349 CCR5 response was detected in both CD8+ and CD4+ T cells. Representative FACS plots demonstrating the responsive cell populations (a) and the sigmoidal concentration dependent responses plotted as curves (b) are shown from two donors.

Figure 4. Concentration dependent inhibition of the pS349 CCR5 response by the small molecule CCR5 inhibitor Maraviroc. Human whole blood was stimulated with

100 ng/ml MIP-1β for 30 minutes in the presence of increasing concentrations of Maraviroc and the pS349 CCR5 response was detected in both CD8+ and CD4+ T cells. Representative FACS plots demonstrating inhibition of the responsive cell populations (a) and the sigmoidal concentration dependent inhibition plotted as curves (b) are shown.

Figure 5. Concentration dependent inhibition of the pS349 CCR5 response by the anti-CCR5 antibody CCR5-MAB1. Human whole blood was stimulated with 100 ng/ml MIP-1β for 30 minutes in the presence of increasing concentrations of CCR5-MAB1 and the pS349 CCR5 response was detected in both CD8+ and CD4+ T cells. Representative FACS plots demonstrating inhibition of the responsive cell populations (a) and the sigmoidal concentration dependent inhibition plotted as curves (b) are shown.

Figure 6. Detection of phosphorylated S349 of CCR5 in cynomolgus and rhesus macaque whole blood. Cynomolgus and rhesus whole blood was stimulated with 100 ng/ml human MIP-1β for 30 minutes and the response in CD8+ and CD4+ T cells was detected with the anti-pS349 CCR5 antibody used to study human responses. Representative data from 2 animals of each species is shown (a). The assay was repeated using multiple donors and the cumulative data compared (N=31) (b). Surface expression of CCR5 was compared to the pS349 CCR5 response in 9 human donors (c).

Figure 1a

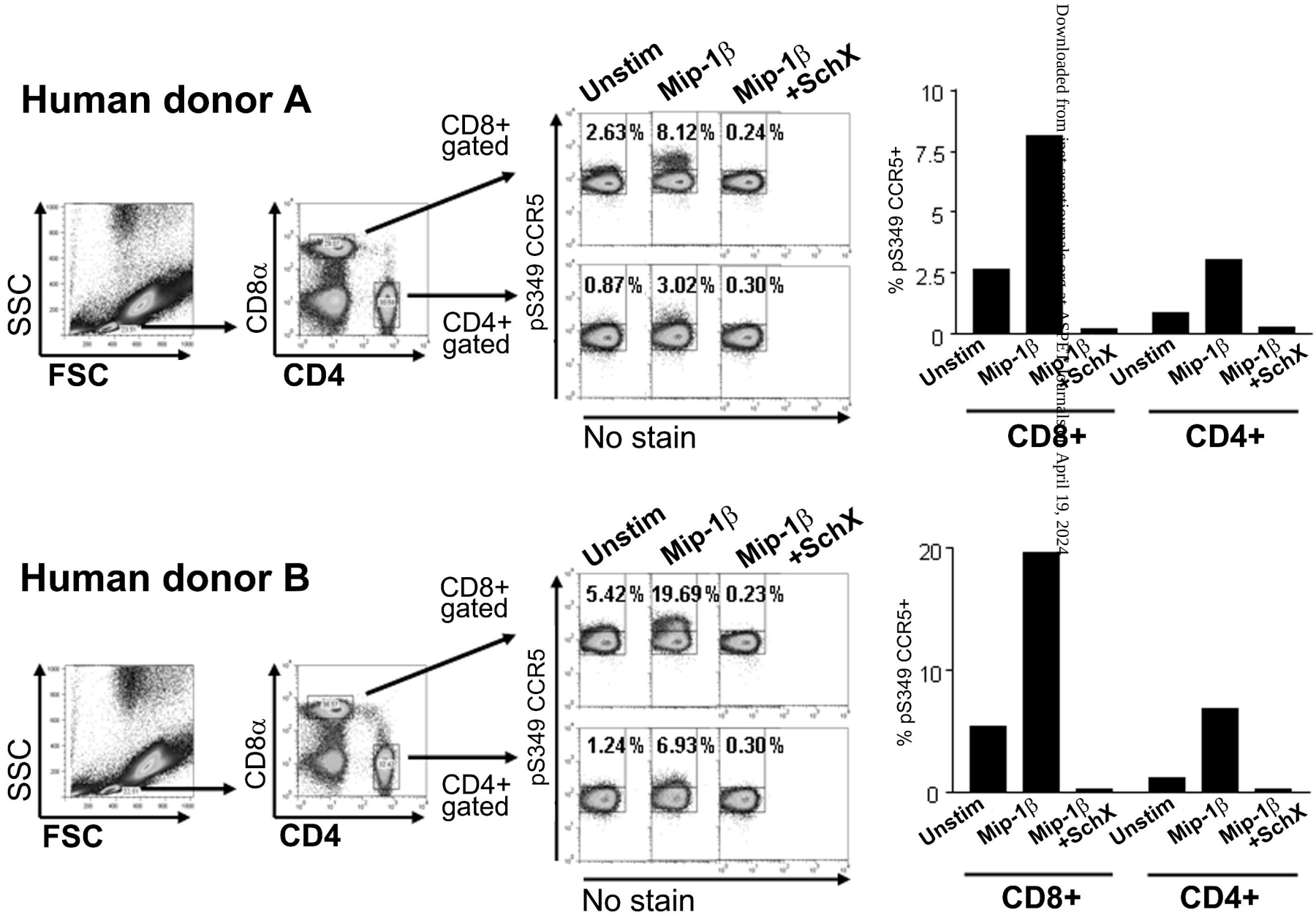


Figure 1b

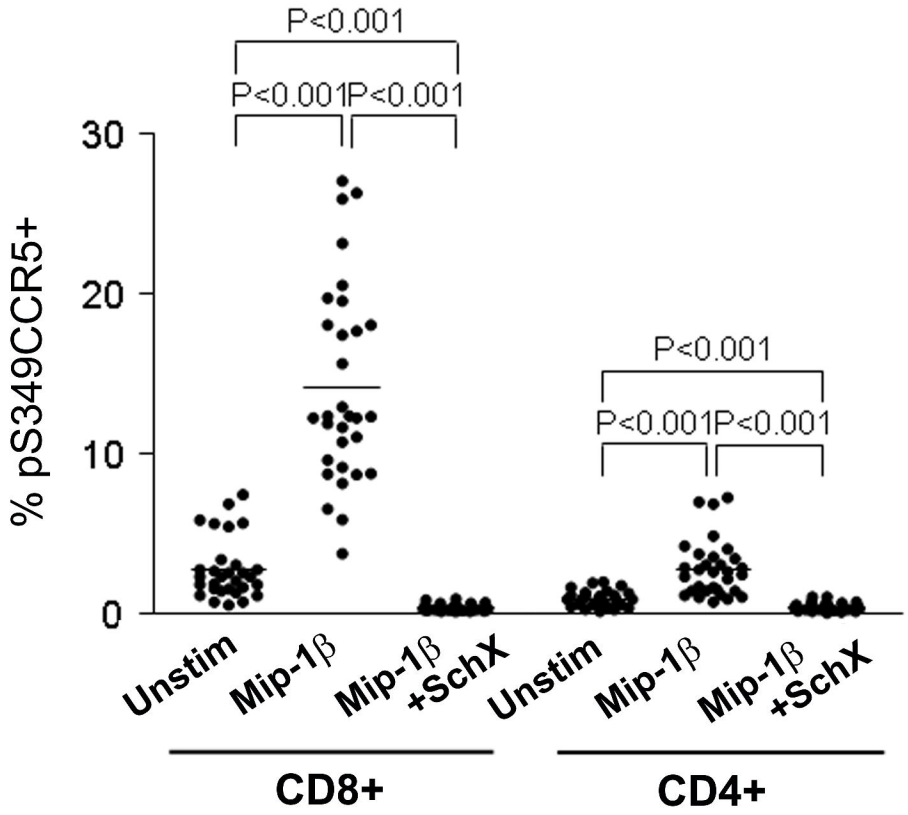
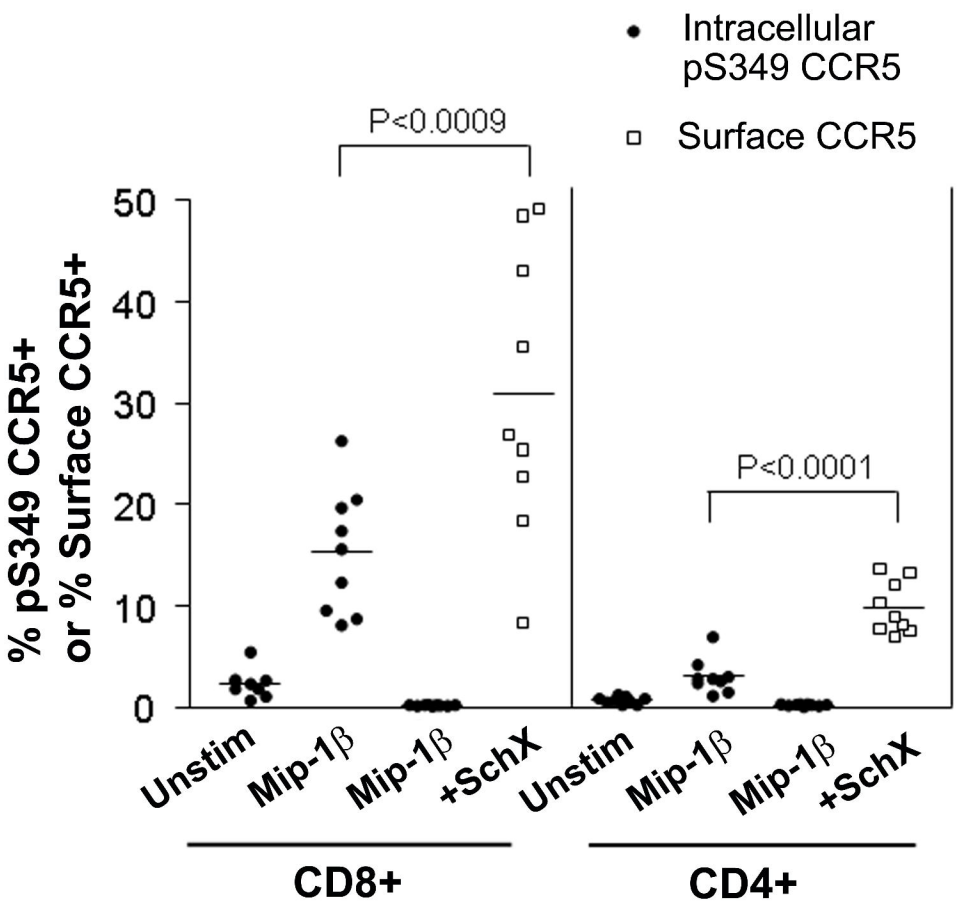


Figure 1c



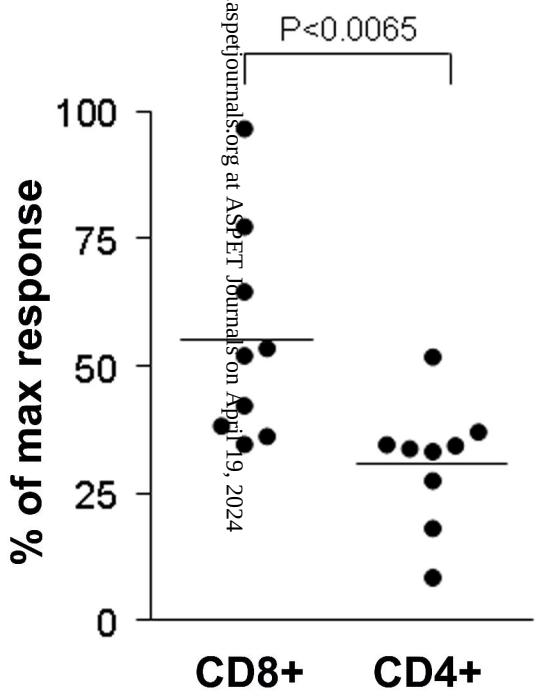


Figure 2

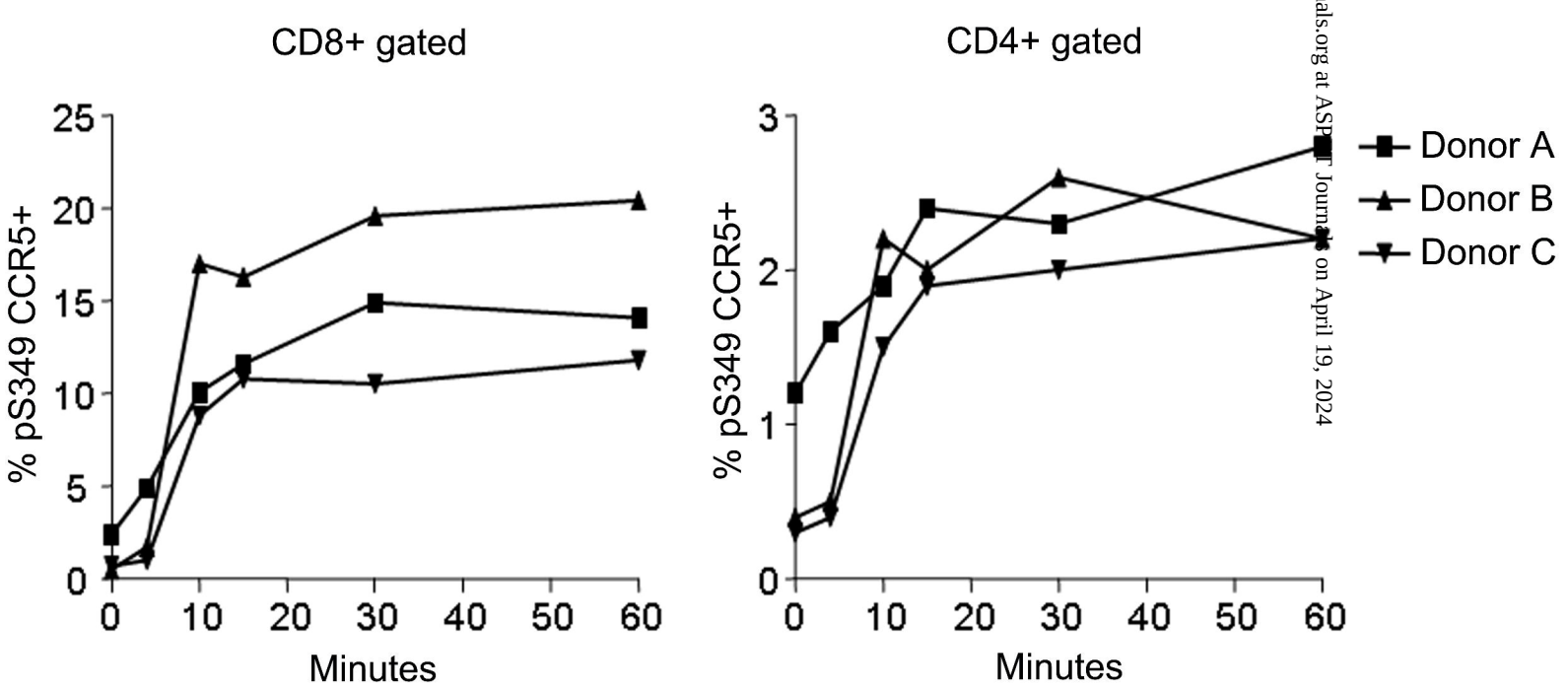


Figure 3a

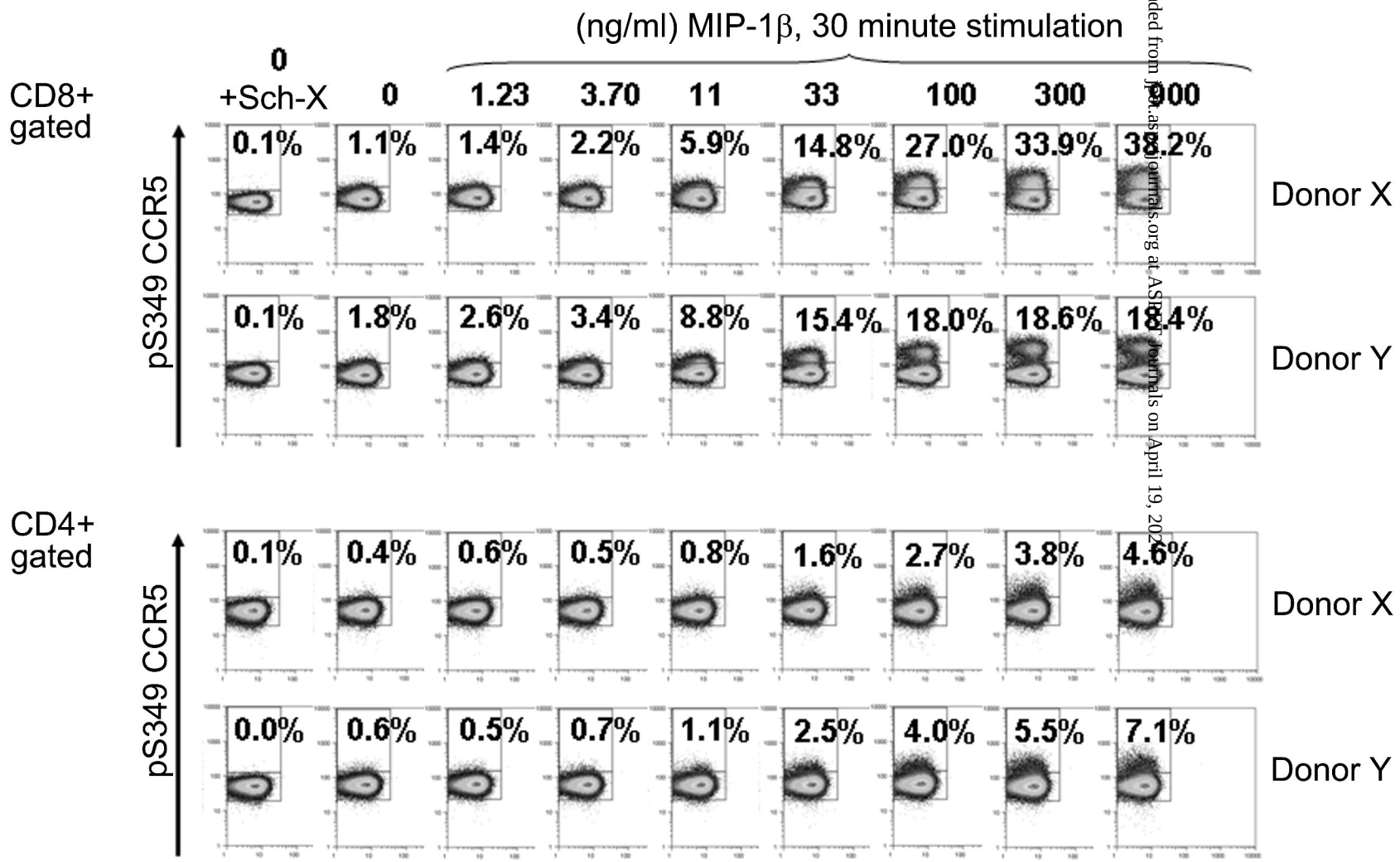


Figure 3b

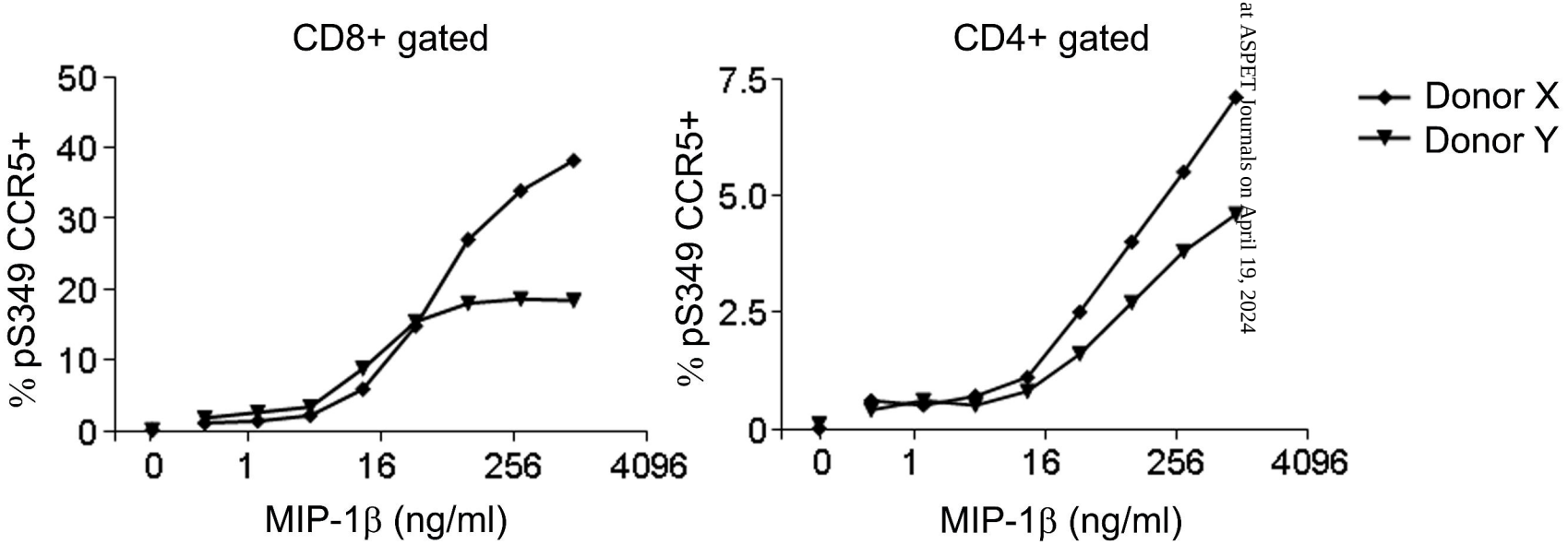


Figure 4a

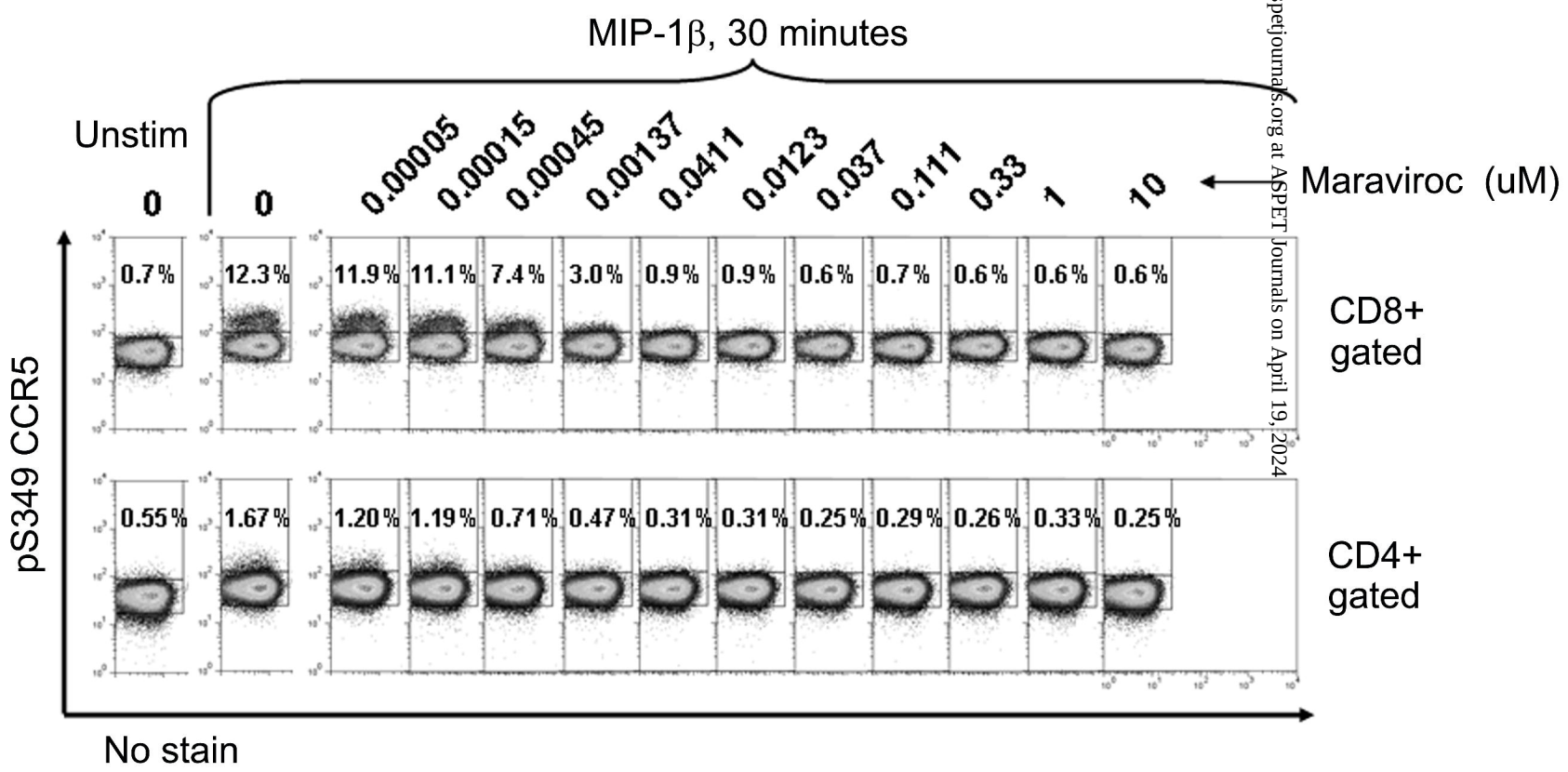


Figure 4b

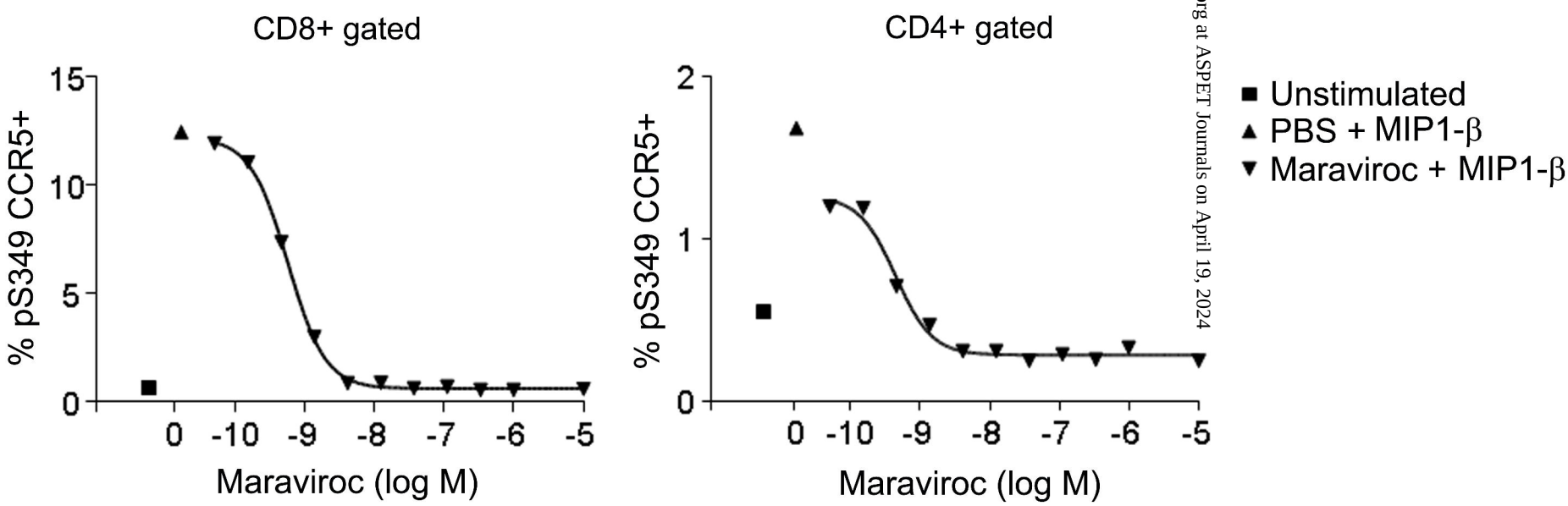


Figure 5a

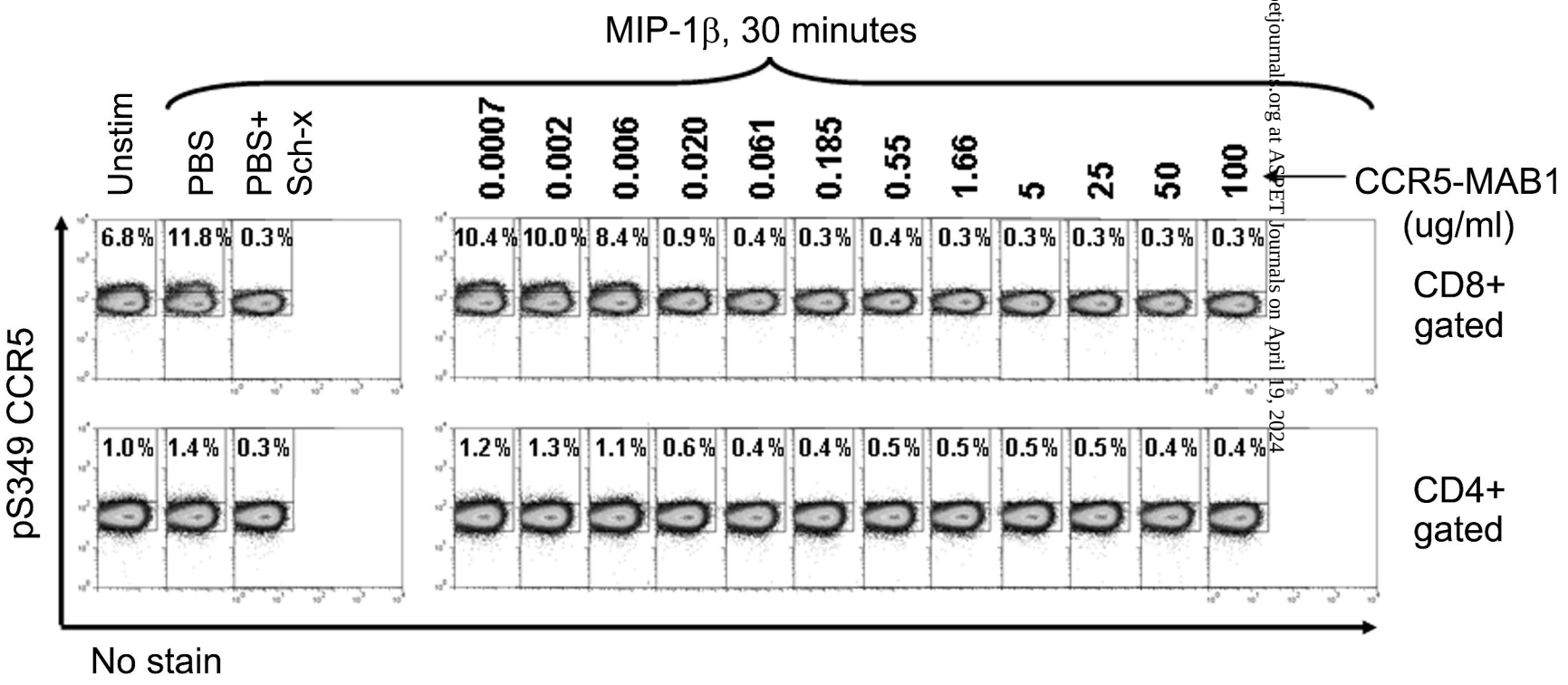


Figure 5b

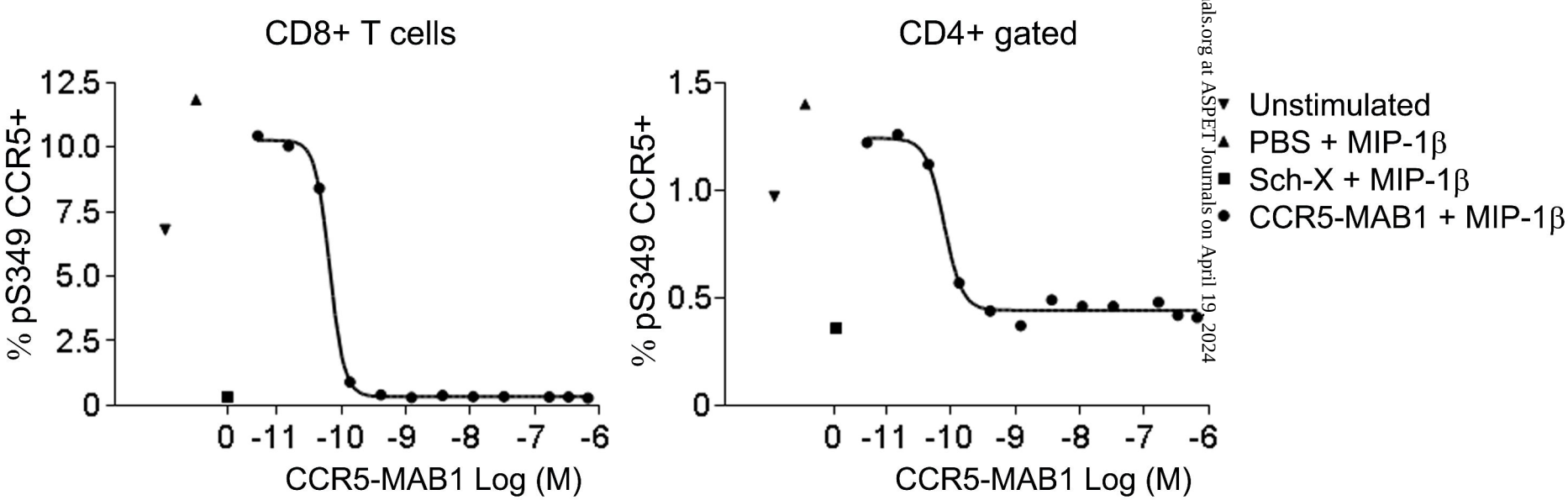
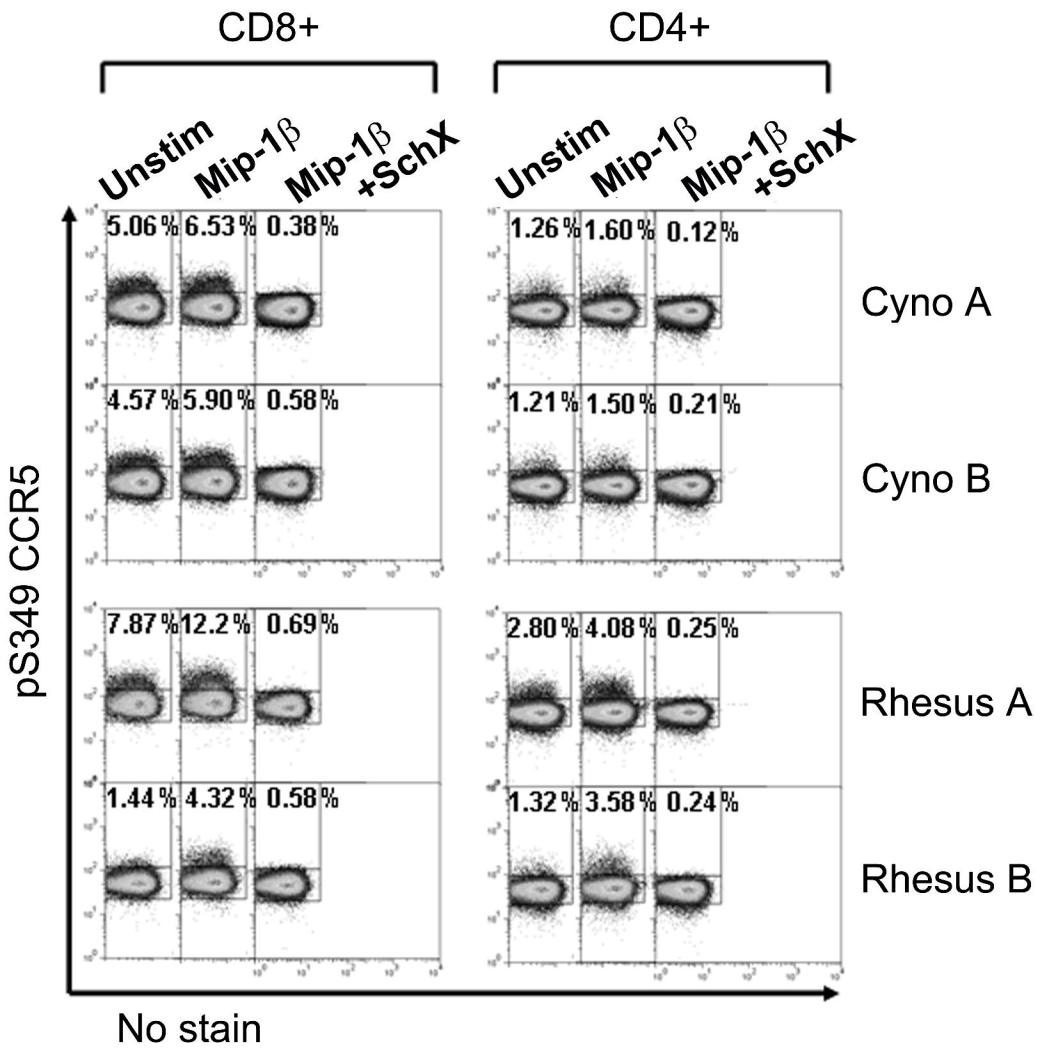


Figure 6a



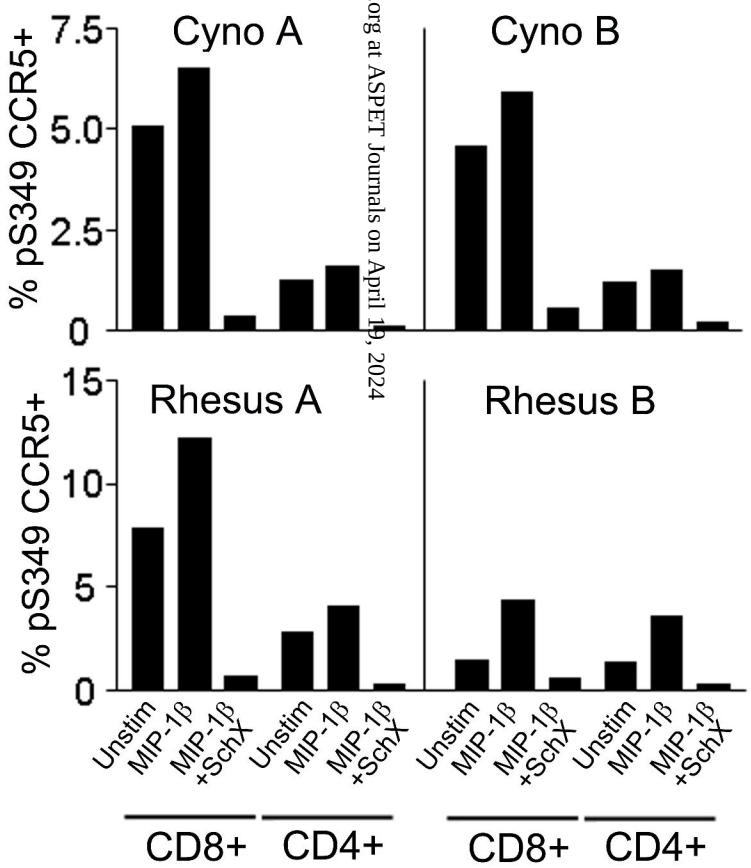


Figure 6b