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Specific MDR1 P-Glycoprotein Blockade Inhibits Human Alloimmune T Cell Activation In Vitro¹

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MDR1 P-glycoprotein (P-gp), the multidrug resistance-associated transmembrane transporter, is physiologically expressed by human peripheral immune cells, but its role in cell-mediated immunity remains poorly understood. Here, we demonstrate a novel role for P-gp in alloantigen-dependent human T cell activation. The pharmacologic P-gp inhibitor tamoxifen (1–10 μ M) and the MDR1 P-gp-specific mAb Hyb-241 (1–20 μ g/ml), which detected surface P-gp on 21% of human CD3⁺ T cells and 84% of CD14⁺ APCs in our studies, inhibited alloantigen-dependent, but not mitogen-dependent, T cell proliferation in a dose-dependent manner from 40–90% ($p < 0.01$). The specific inhibitory effect on alloimmune T cell activation was associated with >85% inhibition ($p < 0.01$) of IL-2, IFN- γ , and TNF- α production in 48-h MLR coculture supernatants. Addition of recombinant human IL-2 (0.1–10 ng/ml) restored proliferation in tamoxifen-treated cocultures. Pretreatment of purified CD4⁺ T cells with Hyb-241 mAb before coculture resulted in inhibition of CD4⁺ T cellular IFN- γ secretion. Also, blockade of P-gp on allogeneic APCs inhibited IL-12 secretion. Taken together these results demonstrate that P-gp is functional on both CD4⁺ T cells and CD14⁺ APCs, and that P-gp blockade may attenuate both IFN- γ and IL-12 through a positive feedback loop. Our results define a novel role for P-gp in alloimmunity and thus raise the intriguing possibility that P-gp may represent a novel therapeutic target in allograft rejection. *The Journal of Immunology*, 2001, 166: 2451–2459.

Overexpression of P-glycoprotein (P-gp),⁴ a 170-kDa transmembrane protein and member of the ATP-binding cassette superfamily of active transporters (1), has been associated with the multidrug-resistant (MDR) phenotype of certain mammalian solid tumors and hematologic malignancies (2, 3). P-gp, the human MDR1 gene product, functions as an ATP-dependent drug efflux pump in such tumors, conferring cellular resistance to cytotoxic xenobiotics by reducing intracellular drug accumulation and, as a consequence, drug toxicity (2, 3). In addition to being overexpressed in cancer cells, P-gp has been found to be widely expressed in normal, predominantly secretory human tissues (4, 5), where it mediates, among other functions, the transmembrane transport of xenobiotics, peptide molecules, certain steroid compounds, and phospholipids (6).

P-gp is expressed by lymphoid cell populations from human bone marrow and peripheral blood. Specifically, P-gp has been shown to be expressed on the membrane of pluripotent stem cells, monocytes, dendritic cells, CD4⁺ and CD8⁺ T lymphocytes, NK

cells, and B lymphocytes (7–16). In mouse peripheral T lymphocytes, expression of P-gp has been reported to identify a subset of activated CD4⁺ T cells characterized by an augmented rate of proliferation and increased IL-2 and IFN- γ secretion in response to various polyclonal stimuli (17). Peripheral T cells derived from P-gp knockout mice, however, were recently found to proliferate and produce cytokines in response to various polyclonal stimuli, and it was suggested that in mice, P-gp is not functional in T cell activation responses (18). In human T cells, P-gp has been reported to facilitate the cellular secretion of several cytokines, including IL-2 and IFN- γ (19, 20). In human myeloid-derived dendritic cells, MDR1 P-gp has been shown to function in transendothelial trafficking and cytokine-dependent mechanisms, possibly involving TNF- α , (21, 22). Hence, despite the provocative evidence for immunoregulatory functions of P-gp, the significance of P-gp expression for the integrity of immunological responses remains uncertain.

While previous studies have focussed on eliciting the function of P-gp in T cell responses to polyclonal stimuli and in cytotoxic T cell function, little is known of the role of P-gp in alloimmune T cell and APC activation responses. However, there is suggestive evidence that P-gp may be important in alloimmunity. Clinico-pathologic studies of human allograft recipients revealed overexpression of P-gp by PBMC (23) and graft tissue-associated mononuclear inflammatory cells (24). Furthermore, P-gp has been reported to be a marker of acute and chronic allograft rejection (23, 24) as well as for therapy-resistant rejection (23). Here we have characterized P-gp expression by human lymphocytes and APCs and have studied the effects of pharmacologic and specific P-gp inhibitors on alloimmune human T cell activation and APC function. We found that pharmacologic and specific P-gp modulators inhibit alloantigen-dependent T cell activation in vitro by blocking IL-2, IFN- γ , and TNF- α release by MLR-stimulated lymphocytes. In addition, P-gp blockade attenuates IL-12 secretion by activated APCs.

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⁴ Abbreviations used in this paper: P-gp, P-glycoprotein; MDR, multidrug-resistant; IC₅₀, 50% inhibitory concentration.

Materials and Methods

Reagents and Abs

Mouse anti-human CD3-PE, anti-human CD4-PE, anti-human CD14-FITC, and anti-human IL-12 p40/p70-PE mAbs were purchased from PharMingen (San Diego, CA). Mouse anti-human TNF- α -PE and PE-conjugated mouse isotype control mAbs were purchased from R&D Systems (Minneapolis, MN). The primary anti-human P-glycoprotein mAb Hyb-241 was a gift from Dr. L. Grauer (Hybritech, a division of Beckman Coulter, San Diego, CA), and the negative control mouse IgG K16/16 (25) was a gift from Dr. M. Gimbrone (Brigham and Women's Hospital, Boston, MA). FITC- and PE-conjugated goat anti-mouse Ig Ab was purchased from Cappel (Durham, NC). Human Ab serum, tamoxifen, LPS, and PHA were purchased from Sigma (St. Louis, MO). Calcein-AM was purchased from Molecular Probes (Eugene, OR).

Cell isolation and culture

PBMCs were isolated by Ficoll-Hypaque gradient centrifugation from blood obtained from healthy volunteers. CD4⁺ T cells were isolated from PBMCs by positive immunomagnetic selection using CD4-coated microbeads (Dynabead CD4/Detachabead CD4-positive isolation kit, Dynal, Lake Success, NY), and CD14⁺ monocytes were isolated from PBMCs by positive selection using CD14-coated microbeads (MiniMACS separation column) purchased from Miltenyi Biotec (Auburn, CA). Cells were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD), supplemented with 10% FCS (Life Technologies, Gaithersburg, MD), 1% penicillin/streptomycin, L-glutamine, and heparin at 37°C in a 5% CO₂ atmosphere.

Analysis of surface marker expression by flow cytometry

To assess P-gp expression of resting lymphocytes and APCs, surface double immunostaining and dual-color flow cytometry of freshly isolated PBMCs were performed following preincubation with buffer containing 20% human serum to block nonspecific Fc receptor binding and optimize specific binding. For indirect staining, 1–2 × 10⁶ PBMCs were incubated for 30 min at 4°C with 5–20 μ g/ml of the primary anti-P-gp mAb Hyb-241 or the negative control mouse IgG mAb, followed by counterstaining with FITC- or PE-conjugated goat anti-mouse Ig Ab. Subsequent direct staining was performed by incubation of indirectly labeled PBMCs with the indicated FITC- or PE-conjugated mAbs for 30 min at 4°C, followed by fixation in 1% paraformaldehyde. Analysis of cell surface marker expression was performed by dual-color flow cytometry using a Becton Dickinson FACScan. When determining lymphocyte- and monocyte-specific surface marker expression by dual-color flow cytometry, selective gating on the respective PBMC subpopulations was performed based on the parameters of forward and side scatter. The percentages of cells positive for PE and/or FITC fluorescence were determined using the CellQuest software package. For the assessment of P-gp expression on PHA- or MLR-stimulated T cells, 2.5 × 10⁵ purified CD4⁺ T cells were cultured in 96-well cell culture plates in a final volume of 200 μ l of culture medium, and 1 μ g/ml PHA or 2.5 × 10⁵ purified allogeneic CD14⁺ APCs were added/well. Indirect surface staining for P-gp using the Hyb-241 mAb was performed as described above, followed by FACS analysis using a Becton Dickinson FACScan.

Fluorescent dye efflux studies

To assess the effects of specific and pharmacologic P-gp inhibition on P-gp-mediated fluorescent dye efflux, 2 × 10⁶ purified CD4⁺ T cells or CD14⁺ APCs were incubated with Hyb-241 mAb (20 μ g/ml), isotype control Ig (20 μ g/ml), or tamoxifen (5 μ M) for 60 min at 37°C in 5% CO₂. Subsequently, calcein-AM was added at a concentration of 1 μ g/ml. After 30 min at 37°C in 5% CO₂, the cells were washed three times with standard culture medium, and serial cell fluorescence measurements were acquired at 0, 30, 60, and 90 min following completion of calcein-AM incubation at the FL2 emission spectrum (585/42 nm) on a Becton Dickinson FACScan.

Intracytoplasmic staining

Intracytoplasmic staining for total IL-12 and TNF- α of LPS-stimulated CD14⁺ monocytes was performed as previously described (26). Briefly, cultured cells were washed in staining buffer (Dulbecco's PBS without Mg²⁺ or Ca²⁺, 0.1% sodium azide, and 1% heat-inactivated FCS) and resuspended in 20% human serum to block Fc receptors. Subsequently, the cells were washed twice in staining buffer, fixed with 0.4% paraformaldehyde, and left for 20 min in the dark. Cells were washed twice and resuspended in permeabilization buffer (Dulbecco's PBS without Mg²⁺ or Ca²⁺, 0.1% sodium azide, 1% heat-inactivated FCS, and 0.1% saponin) and incubated with Abs against cytokines or isotype controls at saturating concentrations for 30 min. Subsequently, the cells were washed in perme-

abilization buffer, then twice in staining buffer, followed by fixation in 1% paraformaldehyde. All staining steps were conducted at 4°C in the dark. Flow cytometry was subsequently performed as described above.

To assess the effect of specific P-gp inhibition on APC cytokine export, purified CD14⁺ cells were seeded at a density of 5 × 10⁵/well in 96-well cell culture plates in a final volume of 200 μ l of culture medium. Cells were preincubated for 60 min with the anti-P-gp mAb Hyb-241 or an isotype control Ab (20 μ g/ml) before addition of 1 μ g/ml LPS. Intracytoplasmic staining was then performed as detailed above, and culture supernatants were harvested for subsequent cytokine analysis by specific ELISA as detailed below.

Cell proliferation and cytokine production assays

Freshly isolated PBMCs were seeded at densities of 5 × 10⁵ or 2.5 × 10⁵/well in 96-well cell culture plates in a final volume of 200 μ l of culture medium, respectively, for use in PHA-stimulated cell proliferation assays and a standard one-way MLR. Cells were preincubated with the pharmacologic P-gp inhibitor tamoxifen (0.1–10 μ M) or the anti-human P-gp mAb Hyb-241 (1–20 μ g/ml) for 60 min before addition of PHA or addition of irradiated (1750 rad) allogeneic stimulator PBMCs (2.5 × 10⁵/well). Cells were cultured at 37°C in 5% CO₂ in the presence of inhibitors. Culture supernatants were taken at 48 h for cytokine analysis by specific ELISA (detailed below), and cell proliferation was assessed after a culture period of 5 days by [³H]thymidine incorporation (1 μ Ci/well) for the last 18 h of culture. Cells were harvested using an automated cell harvester, and incorporated radioactivity was assessed by a Beckman Betamax counter.

In experiments designed to examine whether exogenous IL-2 restored alloantigenic T cell activation in tamoxifen-treated cultures, increasing concentrations (0.1–10 ng/ml) of recombinant human IL-2 (R&D Systems, Minneapolis, MN) were added to MLR cocultures, and cell proliferation was measured by [³H]thymidine uptake on day 5 of culture as described above.

To examine whether P-gp was functional in CD4⁺ T cells, APCs, or both, proliferation and cytokine secretion were studied in cocultures of purified allogeneic CD4⁺ T cells and APCs after preincubation of either cell type with the specific anti-P-gp mAb Hyb-241. Freshly isolated CD4⁺ T cells or purified irradiated (1750 rad) CD14⁺ stimulator APCs were seeded at densities of 2.5 × 10⁵/well (CD4⁺ T cells) or from 2.5 × 10⁴ to 2.5 × 10⁵/well (CD14⁺ APCs) in 96-well cell culture plates in a final volume of 200 μ l of culture medium. Cells were preincubated with the anti-human P-gp mAb Hyb-241 (20 μ g/ml) or isotype control Ig for 60 min at 37°C in 5% CO₂ and then washed twice with standard culture medium. Subsequently, purified irradiated (1750 rad) CD14⁺ stimulator APCs were added to pretreated CD4⁺ T cells at densities from 2.5 × 10⁴ to 2.5 × 10⁵/well. Alternatively, purified allogeneic CD4⁺ T cells (2.5 × 10⁵/well) were added to pretreated CD14⁺ APCs. Coculture supernatants were taken at 48 h for cytokine analysis by specific ELISA, and cell proliferation was assessed after a culture period of 5 days by [³H]thymidine incorporation for the last 18 h of culture.

ELISA

IL-2, IFN- γ , TNF- α , and total IL-12 were assessed by specific ELISAs. Primary and secondary Abs were purchased from Genzyme (Cambridge, MA) and were used according to the recommended protocol. TNF- α and total IL-12 sandwich ELISA kits were purchased from Endogen (Woburn, MA) and used according to the recommended protocol. In brief, 96-well flat-bottom ELISA plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were coated with primary Ab overnight at 4°C. Blocking was then performed with 4% BSA in PBS for 2 h at 37°C, and undiluted culture supernatants or standards were added to each well in duplicate for 1 h at 37°C. After the incubation, secondary biotinylated anti-IL-2, anti-IFN- γ , or anti-TNF- α mAb was added, and the ELISA was developed using avidin alkaline phosphatase (Sigma) and phosphatase substrate (Sigma). In between each step the plates were washed in PBS with 0.01% Triton X-100. Plates were read at 405 nm in an E-Max ELISA plate reader (Molecular Devices, Menlo Park, CA).

Quantification of apoptosis by flow cytometry

Tamoxifen-treated or control MLR-stimulated PBMCs were fixed in ethanol/PBS (70%, v/v, –20°C, 18 h), washed in cold PBS, and then resuspended in propidium iodide stain mixture containing 50 μ g/ml propidium iodide, 0.05% Triton X-100, and 100 U/ml RNase (all purchased from Sigma). DNA content was determined by flow cytometry (Becton Dickinson FACScan) as previously described (27), using CellQuest software.

Trypan blue dye exclusion method for cell viability

Human PBMCs were cultured in the absence or the presence of 10 or 50 μ M tamoxifen at 37°C in 5% CO₂. Following 2-, 12-, and 24-h incubation periods, cells were exposed to 0.4% trypan blue, and the percentage of cells staining blue was counted manually in triplicate samples using light microscopy as previously described (28). The percentage of viable cells was determined according to the formula: % cell viability = 100 - [(no. of blue cells/no. of total cells) \times 100]. A total of 1000 cells were counted in each sample.

Statistical analysis

Results of proliferation, cytokine, and cell viability assays were compared statistically using unpaired two-sided Student's *t* tests (results shown as *p* values). Differences with *p* < 0.05 were considered statistically significant.

Results

Human T cells and APCs express functional surface P-gp

We initially wished to examine the expression of surface P-gp on human T cells and APCs. Double immunostaining and FACS analysis of resting PBMCs, using anti-CD3 or anti-CD14 mAbs, and the Hyb-241 mAb, which recognizes an extracellular P-gp epitope (29), revealed surface P-gp to be expressed on 21% of CD3⁺ T cells and 84% of CD14⁺ APCs (Fig. 1A). Further analysis of purified lymphocyte subpopulations showed specific P-gp staining by 18% of resting CD4⁺ T cells (Fig. 1B). Stimulation of purified CD4⁺ T cells with PHA (1 μ g/ml) or equal numbers of allogeneic CD14⁺ APCs for 24 h did not significantly augment constitutive

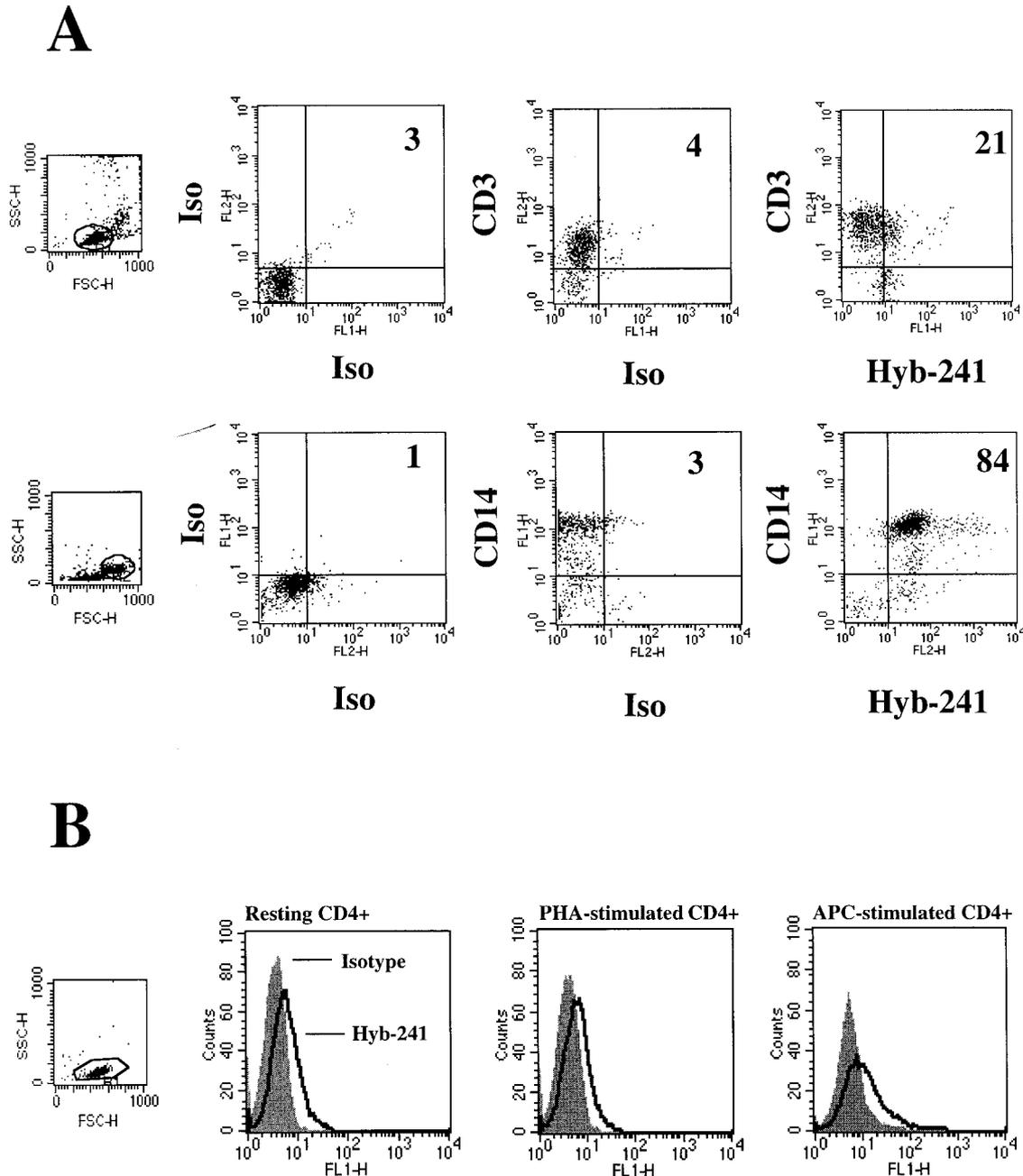


FIGURE 1. Surface P-gp expression of human T cells and monocytes. *A*, Surface P-glycoprotein expression by resting human CD3⁺ T lymphocytes and CD14⁺ monocytes. PBMCs were isolated from healthy human donors and stained for P-gp (using anti-P-gp mAb Hyb-241) and CD3 or CD14. Numbers indicate the percentage of gated cells in respective quadrants. *B*, Surface P-glycoprotein expression by purified CD4⁺ T cells in either the resting state or stimulated for 24 h with PHA (PHA 1 μ g/ml) or allogeneic purified CD14⁺ monocytes. Experiments are representative of three independent experiments. Iso, isotype; FL, fluorescence; FSC, forward scatter; SSC, side scatter.

CD4⁺ T cellular P-gp expression (Fig. 1B). We next examined whether the specific anti-P-gp mAb Hyb-241 and the pharmacologic P-gp inhibitor tamoxifen could inhibit the P-gp-mediated efflux of calcein-AM dye in purified CD4⁺ T cells and CD14⁺ APCs, a characteristic function of P-gp in other cell types (30). Serial fluorometry analyses of calcein-AM-loaded purified CD4⁺ T cells over 90 min following calcein-AM loading revealed a mean cellular fluorescence loss of 47% in untreated or isotype mAb-treated controls vs 15% in tamoxifen (5 μ M)- and Hyb-241 mAb (20 μ g/ml)-treated cultures (Fig. 2). Similarly, tamoxifen (5 μ M) and Hyb-241 mAb (20 μ g/ml) also inhibited calcein-AM efflux in purified CD14⁺ APCs, with enhancements of mean cellular fluorescence of 47 and 28% at 90 min, respectively (data not illustrated). As a control, when cells were kept on ice following calcein-AM incubation to inhibit ATP hydrolysis, no significant dye

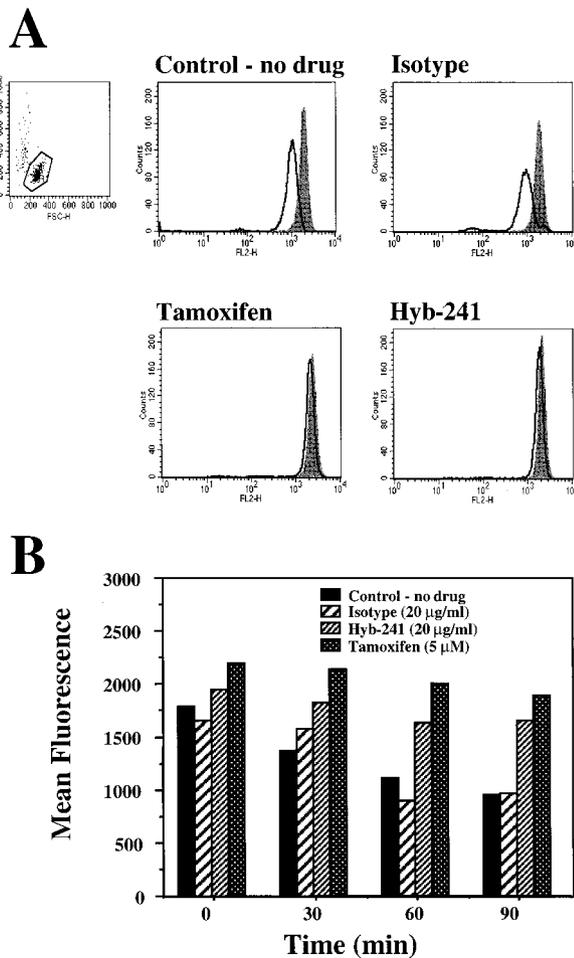


FIGURE 2. Inhibition of calcein-AM efflux of CD4⁺ T cells by pharmacologic and specific P-gp inhibition. *A*, Purified CD4⁺ T cells were incubated with Hyb-241 mAb (20 μ g/ml), isotype control Ig (20 μ g/ml), or tamoxifen (5 μ M) for 60 min at 37°C in 5% CO₂, followed by addition of calcein-AM at a concentration of 1 μ g/ml. Upon 30-min incubation at 37°C in 5% CO₂, the cells were washed three times with standard culture medium, and serial cell fluorescence measurements were subsequently made at 0, 30, 60, and 90 min following termination of calcein-AM incubation at the FL2 emission spectrum (585/42 nm) on a Becton Dickinson FACScan. Cell fluorescence is shown at 0 min (shaded population) and at 90 min (unshaded population) for each untreated and isotype-, Hyb-241 mAb-, or tamoxifen-treated sample. *B*, Mean fluorescence of each untreated or isotype-, Hyb-241 mAb-, or tamoxifen-treated samples is shown vs time (0–90 min) for samples processed as indicated in *A*. Data are representative of three independent experiments.

efflux was observed in control or P-gp inhibitor-treated cultures over a time course from 0–90 min (data not illustrated). Thus, P-gp is expressed on and is active in dye efflux in resting CD4⁺ T cells and CD14⁺ APCs, and is functionally inhibited by the Hyb-241 mAb and the pharmacologic inhibitor tamoxifen.

Tamoxifen inhibits human T cell activation in vitro

We next examined whether P-gp was functional in T cell activation responses. As illustrated in Fig. 3A, we found that tamoxifen

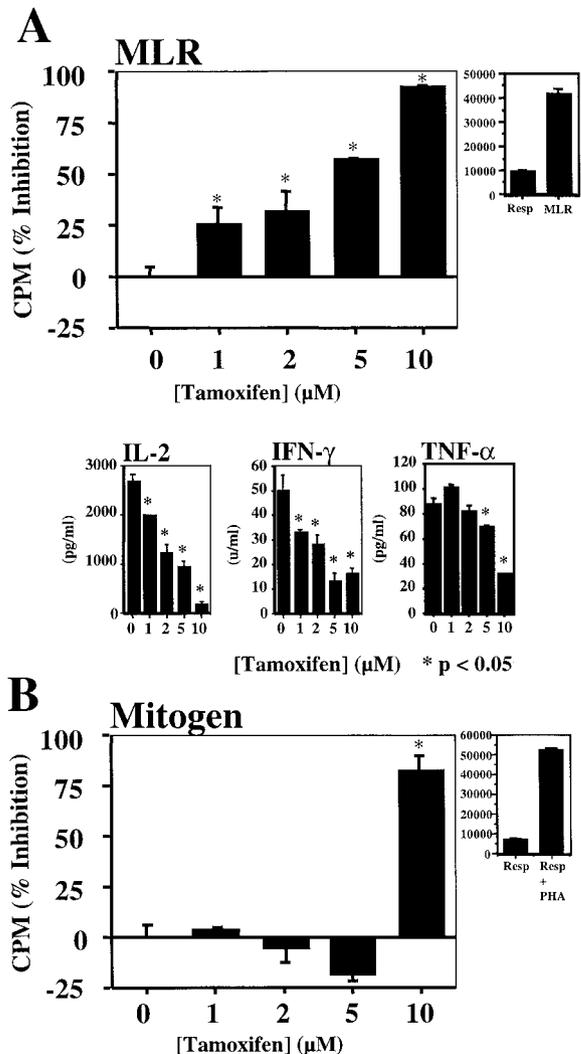


FIGURE 3. Tamoxifen-induced inhibition of human T cell activation. *A*, *Top*, Percent inhibition of [³H]thymidine uptake of tamoxifen-treated alloantigen (MLR)-stimulated human PBMC cultures compared with untreated controls, plotted against the tamoxifen concentration (micromolar). Illustrated are mean values of six separate experiments performed in quadruplicate wells \pm SE. The absolute cpm are illustrated on the *right*. *Bottom*, IL-2 (picograms per milliliter), IFN- γ (units per milliliter), and TNF- α (picograms per milliliter) concentrations determined in 48-h MLR coculture supernatants, plotted against the tamoxifen concentration (micromolar). Illustrated are mean values of triplicate cultures performed in duplicate wells \pm SE. *B*, Percent inhibition of [³H]thymidine uptake of tamoxifen-treated mitogen (1 μ g/ml PHA)-stimulated human PBMC cultures compared with untreated controls plotted against the tamoxifen concentration (micromolar). Mean values of three separate experiments performed in quadruplicate wells \pm SE and absolute cpm are illustrated. Resp, Responder cells.

inhibited alloantigen-dependent T cell proliferation in a dose-dependent manner. Significant inhibition of T cell proliferation occurred at concentrations of tamoxifen as low as $1 \mu\text{M}$ in MLR-stimulated assays ($p = 0.006$), and near-maximal inhibition ($>90\%$) of proliferation was observed at $10 \mu\text{M}$. The 50% inhibitory concentration (IC_{50}) of tamoxifen in alloantigen-dependent assays was observed in the $2\text{--}5 \mu\text{M}$ concentration range. The concentrations of IL-2, IFN- γ , and TNF- α , determined in 48-h coculture supernatants when cell counts are approximately equal, were also reduced in a dose-dependent fashion (Fig. 3A), suggesting a possible cytokine-dependent mechanism for the observed biologic effect. Furthermore, mitogen-dependent T cell proliferation was significantly ($p < 0.001$) inhibited only at higher ($10 \mu\text{M}$) concentrations of tamoxifen (Fig. 3B). T cell proliferation in the MLR assessed on day 5 of culture in the presence of a maximally inhibitory concentration of $10 \mu\text{M}$ tamoxifen was restored by rIL-2 in a dose-dependent fashion, indicating that this biologic effect was not mediated by nonspecific cell killing (Fig. 4). Furthermore, a maximal inhibitory concentration of tamoxifen ($10 \mu\text{M}$) in the MLR did not result in apoptotic cell death above the control value (Fig. 5). In addition, tamoxifen did not induce cell death, as determined by trypan blue dye exclusion, with cell viability percentages ranging from 98 to 100% at $10\text{-}\mu\text{M}$ concentrations of tamoxifen (data not illustrated). This degree of cell viability was comparable to that in untreated controls.

Specific P-gp blockade inhibits alloimmune T cell activation

To address the role of P-gp in alloimmune T cell activation, we next assessed the effects of the P-gp-specific mAb Hyb-241 on T cell activation and proliferation in the MLR. As illustrated in Fig. 6A, Hyb-241 inhibited T cell proliferation in the MLR in a dose-dependent manner, with 40% inhibition at $5 \mu\text{g/ml}$ ($p < 0.001$), and significant inhibition ($p < 0.01$) occurring at mAb concentrations as low as $1 \mu\text{g/ml}$. We measured IL-2, IFN- γ , and TNF- α concentrations in 48-h MLR coculture supernatants and found that

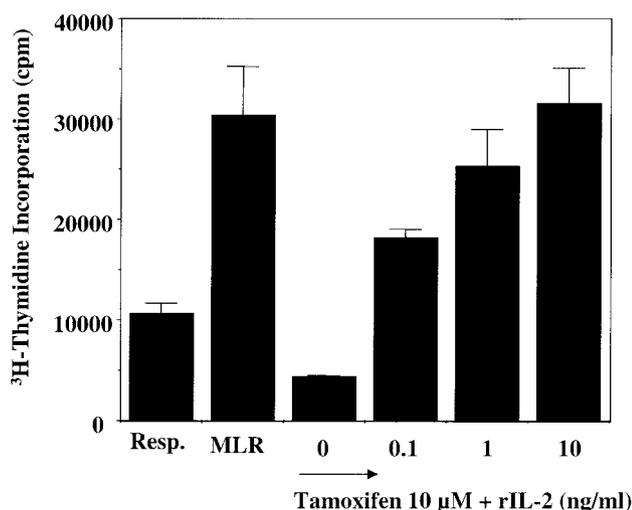


FIGURE 4. Restoration of alloantigen-dependent T cell proliferation in tamoxifen-treated cultures by human rIL-2. Human PBMCs (3×10^5) were cultured alone or with irradiated allogeneic PBMCs (3×10^5) in the absence or the presence of $10 \mu\text{M}$ tamoxifen. Increasing concentrations of human rIL-2 were added to tamoxifen-treated cultures. Proliferation was assessed after 5 days of coculture by [^3H]thymidine incorporation. Illustrated are mean cpm of triplicate cultures \pm SE. Data are representative of three independent experiments. Resp, Responder cells.

inhibition of IL-2 was most pronounced, with $>90\%$ suppression ($p < 0.001$) at Hyb-241 mAb concentrations as low as $1 \mu\text{g/ml}$ (Fig. 6A). Hyb-241 also inhibited cellular IFN- γ and TNF- α in a dose-dependent fashion ($p < 0.001$; Fig. 6A). In contrast to these findings in the MLR, Hyb-241 did not significantly inhibit mitogen-induced T cell proliferation (Fig. 6B).

We next determined whether P-gp was functional via inhibition of T cell activation or/and APC activation. Purified CD4^+ T cells and allogeneic irradiated CD14^+ APCs were cocultured in increasing stimulator-responder ratios after preincubation of either cell type with Hyb-241 mAb. Preincubation of only CD4^+ T cells with Hyb-241 mAb ($20 \mu\text{g/ml}$) resulted in significant inhibition of CD4^+ T cell proliferation by up to 58% at low APC: CD4^+ ratios (1:10; Fig. 7A); increasing the stimulator-responder ratio diminished, but did not abolish, this effect. When irradiated stimulator APCs were preincubated with Hyb-241 mAb ($20 \mu\text{g/ml}$) before coculture, allogeneic CD4^+ T cell proliferation was also significantly inhibited, and this effect varied little with changes in the stimulator-responder ratio (Fig. 7B). IFN- γ secretion was consistently inhibited at all stimulator-responder ratios when CD4^+ T cells were preincubated with Hyb-241 mAb (Fig. 7A). IL-12 concentrations were markedly inhibited in cocultures where APCs had been pretreated with Hyb-241 and to a lesser extent in cocultures following Hyb-241 pretreatment of CD4^+ T cells (Fig. 7, A and B). TNF- α concentrations were also significantly diminished in both assays, but this effect was more pronounced when CD14^+ APCs, rather than CD4^+ cells, had been preincubated with Hyb-241 (Fig. 7, A and B). Thus, P-gp blockade inhibits T cell activation and proliferation by inhibition of both CD4^+ T cell and APC-dependent functions.

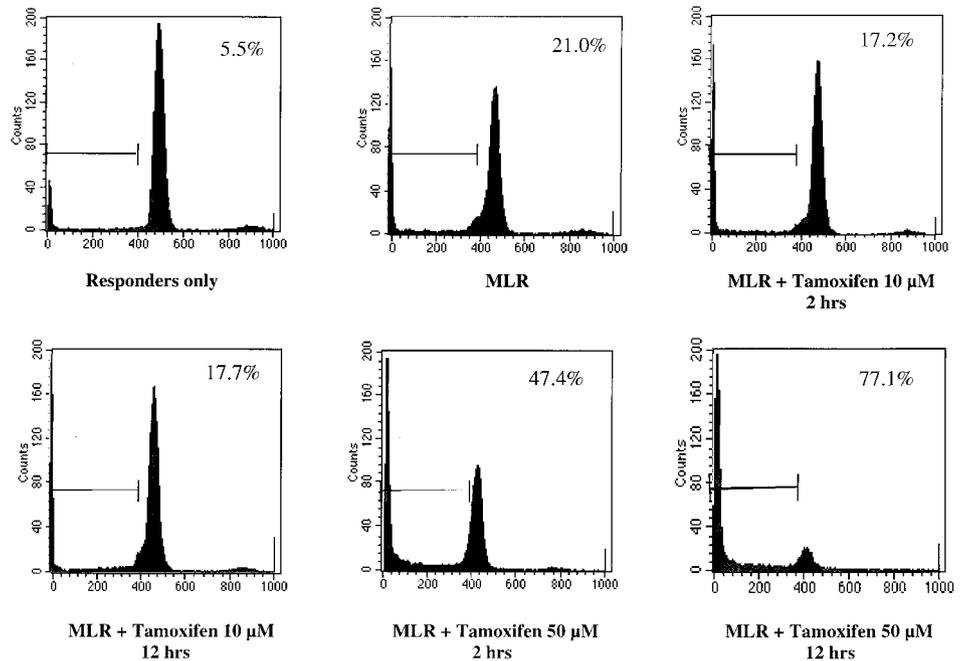
P-gp is functional in monocyte IL-12 secretion

While IL-2, IFN- γ , and IL-4 have been reported to be substrates of P-gp-mediated transport in human T cells, the potential role of P-gp in the transport of other cytokines is currently unknown. To further examine the effects of specific P-gp blockade on TNF- α and IL-12 secretion, we analyzed the effect of specific P-gp inhibition on intracellular TNF- α and IL-12 accumulation in purified cell populations. CD14^+ monocytes were stimulated with LPS in the presence of P-gp inhibition (Hyb-241 mAb), but in the absence of monensin, brefeldin A, or other commonly used inhibitors of cytokine export. We found that Hyb-241 ($20 \mu\text{g/ml}$) treatment did not induce detectable intracellular TNF- α , and secretion of TNF- α by LPS-stimulated monocytes was unaltered in the presence of a maximal inhibitory concentration of Hyb-241 mAb ($20 \mu\text{g/ml}$) compared with control values (Fig. 8, A and B). In contrast, Hyb-241 mAb ($20 \mu\text{g/ml}$), but not an isotype control mAb, did induce intracellular IL-12 accumulation in LPS-stimulated monocytes, while significantly reducing the IL-12 concentration in supernatants of treated cultures (Fig. 8, A and B). This is suggestive that cellular IL-12 transport was inhibited by specific P-gp inhibition.

Discussion

P-gp is physiologically expressed by human immune cells, but its role in cellular immunity is poorly understood. Here, we have examined for the first time a possible function of P-gp in T cellular alloimmunity. Our results reveal that P-gp expressed by human T cells and APCs is functional in alloantigen-dependent human T cell activation in vitro. The expression pattern of P-gp in human T cells reported here is consistent with the findings of previous studies (13, 14). However, the expression and function of surface

FIGURE 5. Assessment of apoptosis by propidium iodide I staining of tamoxifen-treated MLR cultures. Human PBMCs (3×10^5) were cultured alone or with irradiated allogeneic PBMCs (3×10^5) in the presence or the absence of tamoxifen (10 or 50 μM). Cells were examined at the indicated time points by PI staining as described in *Materials and Methods*. A leftward shift in PI staining indicates DNA fragmentation and cellular apoptosis. The percentage of apoptotic cells in the total cell population of each sample is shown in the *upper right corner* of each panel. Apoptosis was not induced in the presence of functional concentrations of tamoxifen (10 μM). However, toxic concentrations of tamoxifen (50 μM) did induce apoptosis and are illustrated as a positive control.



P-gp by the majority of CD14⁺ cells is different from several previous reports illustrating low P-gp expression on monocytes (10, 11, 14). Our findings, however, are consistent with one report that used another P-gp-specific mAb (MRK16) (31). Moreover, our finding of functional inhibition of calcein-AM dye efflux from purified CD4⁺ T cells and monocytes by specific (Hyb-241 mAb) and pharmacologic P-gp inhibitors, a well-described function of P-gp in other cell types (30, 32), confirmed that P-gp is functional in CD4⁺ T cells and monocytes.

To assess the functional significance of P-gp expression in T cells and APCs, we examined the effects of tamoxifen, a nonsteroidal triphenylethylene compound and known pharmacologic P-gp inhibitor (33), on T cell activation responses. Tamoxifen inhibited alloantigen-dependent, but not mitogen-dependent, T cell proliferation in a dose-dependent manner. While tamoxifen is known to exert pleiotropic cellular effects (33), the absence of tamoxifen-induced apoptosis or nonspecific cell death and the coincidence of the observed IC₅₀ with the known IC₅₀ of tamoxifen for inhibition of P-gp (34) suggest that this novel inhibitory effect of tamoxifen on alloimmune T cell activation was mediated by inhibition of P-gp. Our finding that tamoxifen inhibits IL-2 and IFN- γ in supernatants of MLR cocultures, two cytokines that have been implicated as potential substrates of P-gp-mediated transport (19, 20), and our demonstration of tamoxifen-induced inhibition of P-gp transport function on CD4⁺ T cells and APCs are consistent with this interpretation. To specifically address the role of P-gp in cell-mediated alloimmunity, we used the Hyb-241 mAb, which is known to be specific for MDR1 P-gp (29). Hyb-241 is a known inhibitor of P-gp function (35) and in our studies binds P-gp on both human T cells and APCs. Furthermore, our studies provide direct evidence that Hyb-241 inhibits P-gp-mediated transport functions at concentrations that also inhibited alloimmune T cell activation. Our findings of specific inhibition of alloantigen-dependent, but not mitogen-dependent, T cell proliferation by Hyb-241 and of diminished IL-2, IFN- γ , and TNF- α concentrations in supernatants of MLR cocultures provide evidence for a functional role for P-gp in alloimmune T cell activation. The absence of effects of specific P-gp inhibition on human T cell proliferation in response to mitogen-induced activation is consistent with the re-

cent demonstration of normal proliferation and cytokine secretion of murine T cells derived from *mdr1a* gene knockout mice in response to polyclonal stimuli (18).

Based on its known function as a transmembrane transporter (22), P-gp has been proposed to facilitate the transmembrane transport and/or release of cytokines. The possible role of P-gp in T cellular secretion of certain cytokines has been addressed by several investigators, but the results of these studies have been controversial. Two publications have suggested that P-gp is involved in the release of IL-2, IL-4, and IFN- γ from human PBMCs (19, 20). T cellular P-gp expression, however, was not directly demonstrated in these studies, and the proposed function was in part inferred from experiments using nonspecific, pharmacologic P-gp inhibitors. Other authors failed to demonstrate P-gp expression on IL-2-secreting human lymphocytes and suggested that P-gp does not play a major role in IL-2 secretion by activated T cells (36). In addition, recent studies have demonstrated that lymphocytes derived from *mdr1a* gene knockout mice exhibit normal cytokine production in response to polyclonal activation (18).

We found that the Hyb-241 mAb exerts an inhibitory effect on human CD4⁺ T cells and CD14⁺ monocytes. Specific P-gp blockade of CD4⁺ T cells alone resulted in diminished IFN- γ secretion by CD4⁺ alloreactive T cells, consistent with the previously postulated role of P-gp in the cellular transport of this cytokine (19). In addition, we found evidence for a role of P-gp in the cellular export and/or secretion of IL-12 by activated CD14⁺ monocytes. Our results suggest that P-gp blockade may dysregulate the IFN- γ /IL-12-positive feedback loop by inhibiting cellular functions of both the CD4⁺ T cell and the APC, resulting in attenuation of Th1-type cytokine responses. It is also possible that CD4⁺ P-gp blockade inhibited the production of an unidentified CD4⁺ T cell-derived factor(s), which augments monocyte IL-12 production. An interesting finding of our studies is that a brief incubation of T cells or APCs with the anti-P-gp Ab before the MLR was begun maintained inhibition for the 5-day duration of the MLR. This implies that the role of P-gp is manifested early, since it is unlikely, especially in the case of the APC, that any residual Ab remained on these cells after prolonged culture. This finding is consistent with

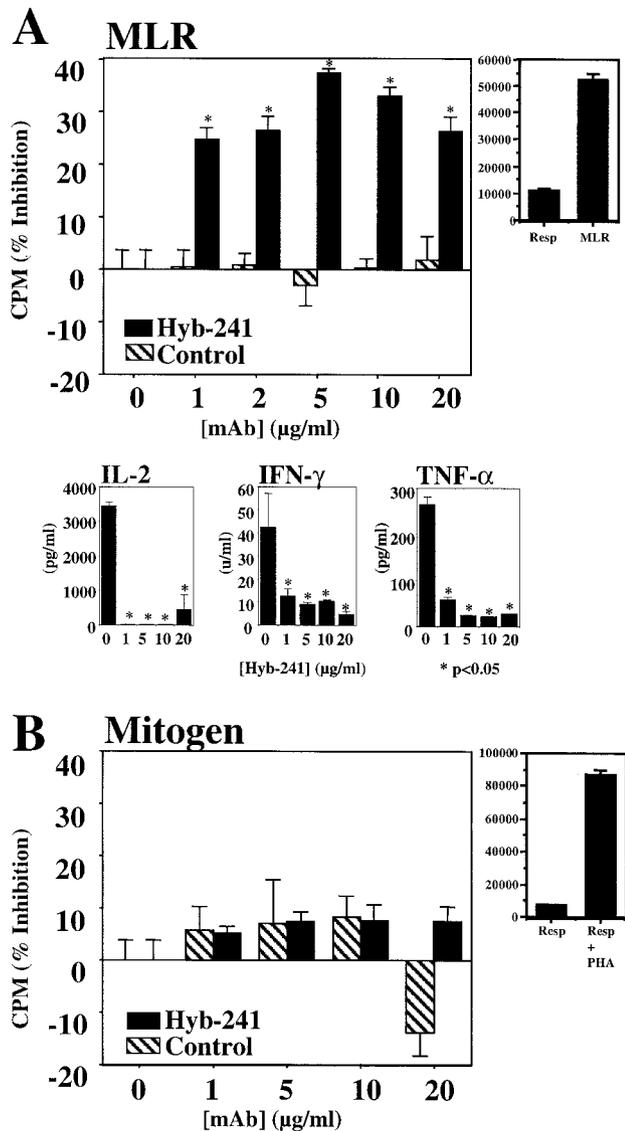


FIGURE 6. Inhibition of alloantigen-dependent T cell activation by specific P-gp blockade. *A, Top*, Percent inhibition of [^3H]thymidine uptake (cpm) of anti-P-gp mAb (Hyb-241)-treated alloantigen (MLR)-stimulated human PBMC cultures compared with untreated controls, plotted against the Hyb-241 mAb concentration (micrograms per milliliter). The absolute cpm values are illustrated on the *right*. An isotype control mAb had no significant inhibitory effect. Illustrated are mean values of quadruplicate cultures \pm SE. *Bottom*, IL-2 (picograms per milliliter), IFN- γ (units per milliliter), and TNF- α concentrations (picograms per milliliter) determined in anti-P-gp mAb (Hyb-241)-treated 48 h MLR coculture supernatants, plotted against the Hyb-241 mAb concentration (micromolar). Illustrated are mean values of triplicate cultures performed in duplicate wells \pm SE. *B*, Percent inhibition of [^3H]thymidine uptake of anti-P-gp mAb (Hyb-241)-treated mitogen (1 $\mu\text{g/ml}$ PHA)-stimulated human PBMC cultures compared with untreated controls, plotted against the Hyb-241 mAb concentration (micrograms per milliliter). The absolute cpm are illustrated on the *right*. An isotype control mAb had no significant inhibitory effect. Illustrated are mean values of quadruplicate cultures \pm SE. Data in *A* and *B* are representative of three independent experiments. Resp, Responder cells.

the results found by Randolph et al. that preincubation of monocytes with anti-P-gp mAb inhibited reverse migration of dendritic cell precursors for up to several days (22).

In our studies P-gp inhibition reduced TNF- α production in the MLR. In contrast, no significant effect was observed on the secre-

tion of TNF- α by LPS-activated monocytes in the presence of specific P-gp inhibition. This finding suggests that suppressed TNF- α production in alloimmune-stimulated cultures is mediated by P-gp inhibition of cellular activation rather than cellular TNF- α transport. In addition, we used purified monocytes in our LPS stimulation assays such that these cells had not differentiated into efficient APCs (dendritic cells) as in the MLR. Thus, the effect of P-gp may also be on these differentiated cells in the MLR and not on pure populations of CD14 $^+$ monocytes. Alternatively, P-gp may be functional in TNF production by lymphocytes, but since APCs are the main source of TNF in the MLR, we favor an effect on the APC.

In addition to the cytokine-dependent mechanisms identified in our studies and by others, P-gp may be functional in alloimmunity in Ag processing and/or in Ag presentation. Two pathways of allorecognition have been described. In the direct pathway, T cells recognize intact allo-MHC molecules on the surface of donor allogeneic cells. In the indirect pathway, T cells recognize processed alloepitope in a self-restricted manner presented by self-APCs (37). P-gp is a functional homologue of TAP (38), which is critical in the assembly and expression of peptide-class I MHC complexes (39). While P-gp is not involved in class I MHC-associated presentation to cytolytic CD8 $^+$ T lymphocytes (38), the role of this transporter or a similar P-gp-regulated transporter of class II MHC-associated molecules for presentation to CD4 $^+$ T cells has not been described. Our finding of high P-gp expression by CD14 $^+$ APCs and of specific inhibition of alloantigen-dependent but not mitogen-dependent T cell activation by the anti-P-gp mAb Hyb-241 would be consistent with such a function of the molecule. However, our finding that alloimmune T cell activation was also inhibited when CD4 $^+$ T cellular P-gp was selectively blocked suggests that such a role of P-gp as a primary mechanism in alloimmunity is unlikely.

Lastly, it is possible that P-gp serves a role in the regulation of cellular volume/shape changes, which could dysregulate cell/cell interactions and the efficacy of stimulatory and costimulatory processes. P-gp has been shown to regulate a ubiquitously expressed volume-regulated chloride channel activity (40, 41) associated with cellular volume adaptations in certain cell types. This P-gp-associated volume-regulated chloride channel is functionally blocked by P-gp-specific mAbs (42, 43) and MDR1 antisense oligonucleotides (44). A molecular candidate for the volume-regulated chloride channel, CIC-3, has recently been identified, and was shown to be inhibited by the chloride channel-specific inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid and the P-gp inhibitor tamoxifen (45). Since T cell activation depends in part on efficient cell-cell interactions during Ag recognition and costimulation, P-gp, through its associated chloride channel activity (46), may function as a regulator of cellular volume changes and cell shape adaptations during these processes. The recent observation of inhibition of immunological (47, 48) and cell proliferative (49) responses by chloride channel blockade supports this possibility, but whether such mechanisms contribute to P-gp function in alloimmunity identified in this study has not been established.

In summary, our results demonstrate for the first time that P-gp is functional in human alloantigen-dependent T cell activation. P-gp is functional on both CD4 $^+$ T cells and APCs, and blockade of P-gp inhibits select responses in both cell types. We believe that these findings point to a novel and specific functional role of P-gp in alloimmunity. P-gp may thus represent a novel therapeutic target in allograft rejection.

FIGURE 7. Selective effect of specific P-gp blockade on CD4⁺ T cells and CD14⁺ APCs. *A, Top*, CD4⁺ T cells were preincubated with Hyb-241 mAb (20 μ g/ml) or isotype control Ig (20 μ g/ml), followed by washing and subsequent stimulation with increasing numbers of allogeneic purified CD14⁺ APCs. *B, Top*, CD14⁺ APCs were preincubated with Hyb-241 mAb (20 μ g/ml) or isotype control Ig (20 μ g/ml), followed by washing and subsequent stimulation with allogeneic purified CD4⁺ T cells. For the experiments illustrated in *A* and *B*, proliferation is plotted against the APC:CD4⁺ cell ratio for isotype control-treated cultures (●) and Hyb-241-treated cultures (○). The mean values of quadruplicate cultures \pm SE are shown. *A* and *B, Bottom*, IFN- γ (units per milliliter), IL-12 (picograms per milliliter), and TNF- α (picograms per milliliter) concentrations determined in 48-h supernatants of APC/CD4⁺ T cell cocultures as described above, plotted against the APC:CD4⁺ cell ratio. Illustrated are mean values of triplicate cultures performed in duplicate wells \pm SE. The isotype control mAb had no significant inhibitory effect vs untreated controls. For all illustrated data, similar results were obtained in three separate experiments.

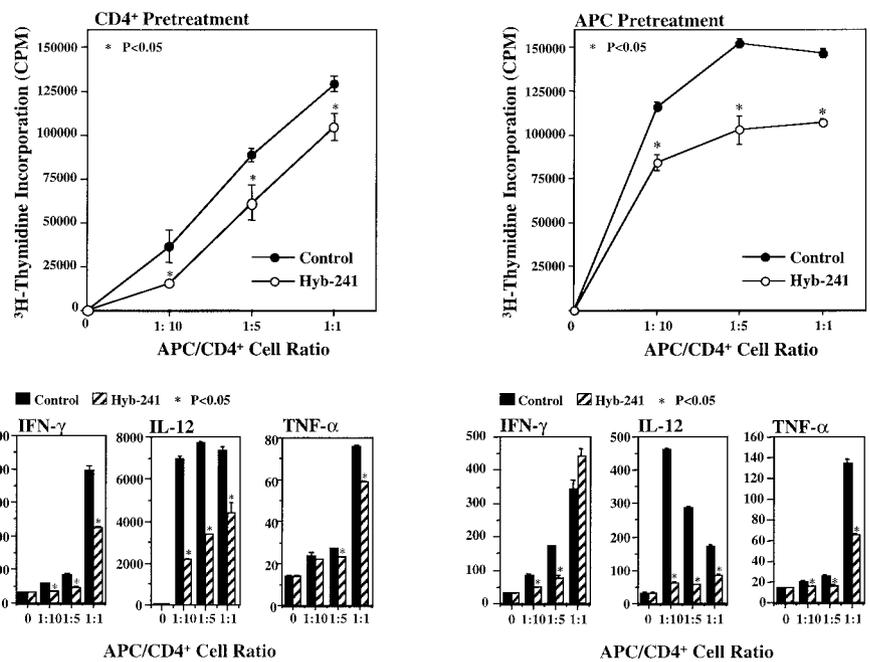
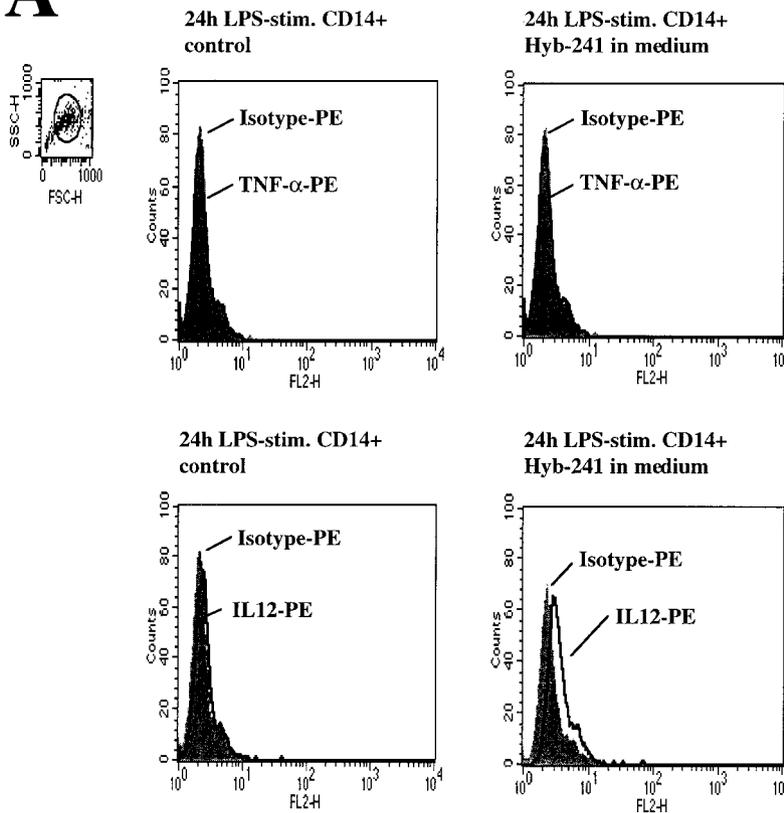
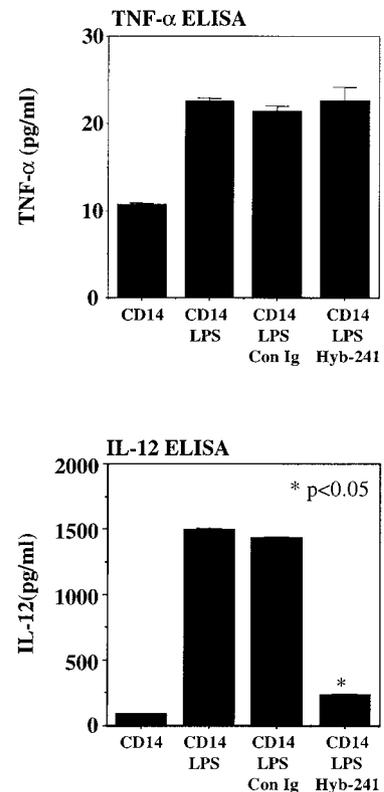
**A****B**

FIGURE 8. Intracellular TNF- α and IL-12 staining of LPS-stimulated monocytes and cytokine analysis of culture supernatants in the presence of P-gp blockade. *A*, Purified CD14⁺ monocytes were stimulated with LPS (1 μ g/ml) for 24 h in the presence of Hyb-241 mAb (20 μ g/ml) or isotype control Ig (20 μ g/ml). Subsequently, intracellular staining was performed for TNF- α and IL-12, followed by FACS analysis. The shaded population represents isotype-PE staining; the unshaded populations represent TNF- α -PE staining (*top*) or IL-12-PE staining (*bottom*). Illustrated are typical results of three independent experiments. *B*, TNF- α (picograms per milliliter; *top*) and IL-12 (*bottom*) concentrations determined from culture supernatants of purified CD14⁺ APCs stimulated with LPS (1 μ g/ml) for 24 h in the presence of Hyb-241 mAb (20 μ g/ml) or isotype control Ig (20 μ g/ml). Illustrated are mean values of triplicate cultures \pm SE. FL, fluorescence; FSC, forward scatter; SSC, side scatter.

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