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# The Multidrug Resistance Protein 1: A Functionally Important Activation Marker for Murine Th1 Cells<sup>1</sup>

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Previously, we described the expression of an energy-dependent pump in resting murine Th2 (but not resting Th1) cells which extruded the fluorescent dye Fluo-3. After stimulation with Ag and APCs, Th1 cells also expressed this pump. Furthermore, expression of the murine multidrug resistance protein 1 (mrp1) correlated with the presence of the pump. In this study, we report that Fluo-3 is indeed transported by murine mrp1 or its human ortholog MRP1, as revealed by transfection of HEK 293 cells with mrp1 or MRP1 cDNA. Like antigenic activation, IL-2 dose-dependently enhanced the Fluo-3-extruding activity in murine Th1 cells. Although TNF- $\alpha$  and IL-12 by themselves only weakly enhanced Fluo-3 extrusion, each of them did so in strong synergism with IL-2. An Ab directed against mrp1 was used to quantify the expression of mrp1 protein in T cells at the single-cell level. Like the Fluo-3 pump, mrp1 protein expression was enhanced by IL-2. Immunohistochemical studies using confocal laser microscopy indicated that mrp1 is localized mainly at the plasma membrane. In addition, protein expression of mrp1 was induced in V $\beta$ 8<sup>+</sup>CD4<sup>+</sup> T cells 12 h after in vivo application of Staphylococcal enterotoxin B. Finally, mrp1 was functionally relevant during the activation process of Th1 cells, because T cell activation could be suppressed by exposure of cells to the mrp1 inhibitor MK571. Thus, we present mrp1 as a novel, functionally important activation marker for Th1 cells and short-term in vivo activated CD4<sup>+</sup> T cells, whereas its expression seems to be constitutive in Th2 cells. *The Journal of Immunology*, 2000, 164: 754–761.

Murine CD4<sup>+</sup> T lymphocytes can be subdivided mainly into two phenotypically distinct subpopulations termed Th1 and Th2 cells (1, 2). The two subpopulations differ with respect to the cytokine pattern they produce: Th1 cells secrete IL-2, IFN- $\gamma$ , and TNF- $\beta$ , whereas the Th2 cytokine repertoire consists of IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (3). Th1 and Th2 cells act as regulators and activators of the immune response and trigger the immune system toward a macrophage-dominated type 1 or a B cell-dominated type 2 response, respectively. Several in vivo models have been generated for studying the differences between Th1- and Th2-mediated immunity. One of them, experimental cutaneous murine leishmaniasis, caused by s.c. infection with the protozoan parasite *Leishmania major*, leads to a Th1 response in resistant C57BL/6 mice and to a Th2 response in susceptible BALB/c mice defined by the respective cytokine profile (4–7).

Such in vivo models have prompted the search for specific surface molecules which would facilitate identification of the two CD4<sup>+</sup> T cell subsets. In this regard, it was recently shown that the IL-18 receptor is selectively expressed on the surface of Th1 cells (8). On the other hand, Th2 cells could be detected with the help of the cell surface molecule ST2L (9) as well as by high expression of CD30 (10). Similarly, chemokine receptors were shown to be

useful markers for the discrimination of T cell subsets. For instance, the chemokine receptors CXCR3 and CCR5 were reported to be mainly expressed on the surface of Th1 cells, although this finding was only partially confirmed by others (11–13). Abs to the eotaxin receptor CCR3 were used for isolation of Th2, but not Th1 cells from human peripheral blood (14). The highest preference for Th2 lymphocytes was shown by the chemokine receptor CCR4 (11, 13).

Recently, we have described (15) another candidate for Th1 and Th2 discrimination, namely, a transmembrane transporter molecule. The protein exports the fluorescent anion Fluo-3 out of the cytoplasm of T cells (16) and is differentially expressed in resting Th1 and Th2 cells: resting Th2 cells transport Fluo-3 whereas Th1 cells are unable to do so. Stimulation of Th1 cells with Ag and APCs results in an up-regulation of the anion transporter comparable to resting Th2 cells. Northern blotting and dye extrusion inhibition studies in resting Th1 and Th2 cells raised the possibility that this pump might be murine multidrug resistance protein 1 (mrp1),<sup>3</sup> the ortholog of the human MRP1. Indeed, in one Th1 cell clone, mrp1 protein was up-regulated after antigenic restimulation like the Fluo-3 pump, as revealed by immunoblot analysis.

MRP1 is a member of the family of ATP-binding cassette transporters that has been shown to mediate multidrug resistance in tumor cells. It is also expressed in normal tissues, including several types of epithelia, muscle cells, and macrophages (17, 18). Low levels of MRP mRNA are expressed in normal peripheral blood CD4<sup>+</sup> T cells (19).

In the present investigation, we further studied the relation of mrp1 and the Fluo-3 pump and their expression in CD4<sup>+</sup> T cells.

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<sup>3</sup> Abbreviations used in this paper: mrp1, murine multidrug resistance protein 1; Fluo-3-AM, fluo-3-acetoxymethyl ester; CsA, cyclosporin A; LTC<sub>4</sub>, leukotriene C<sub>4</sub>; MRP1, human MRP1; PPD, purified protein derivative; rm, recombinant murine; SEB, *Staphylococcus aureus* enterotoxin B; SN, supernatant.

We directly show that mrp1 exports Fluo-3, the cleaved intracellular fluorescent anion of the acetomethyl ester fluo-3-acetoxymethyl ester (Fluo-3-AM). In addition, we demonstrate at the single-cell level that not only antigenic activation, but also cytokine stimulation, enhances Fluo-3 extrusion as well as mrp1 expression in Th1 cells. Finally, we present evidence that mrp1 activity is a prerequisite for activation of Th1 cells by Ag or cytokines.

## Materials and Methods

### Reagents

Fluo-3-AM, Triton-X-100, paraformaldehyde, sodium citrate, FCS, *Staphylococcus aureus* enterotoxin B (SEB), and saponin were purchased from Sigma (Deisenhofen, Germany). Cyclosporin A (CsA) was purchased from Calbiochem (La Jolla, CA). MK571 was purchased from Biomol (Hamburg, Germany). PSC 833 was purchased from Novartis (Nuremberg, Germany). The cytokines TNF- $\alpha$ , IL-2, and IL-12 were purchased from PharMingen (Hamburg, Germany). For ELISA studies, commercial Ab pairs from PharMingen were used.

### Treatment of mice

Female BALB/c mice at the age of 4–6 wk were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). They were injected with 10  $\mu$ g of SEB in 50  $\mu$ l of PBS into the hind footpad. Control mice were injected with 50  $\mu$ l of PBS only. After 12 h, the draining popliteal lymph nodes were removed and a single cell suspension was prepared for flow cytometry.

### T cells

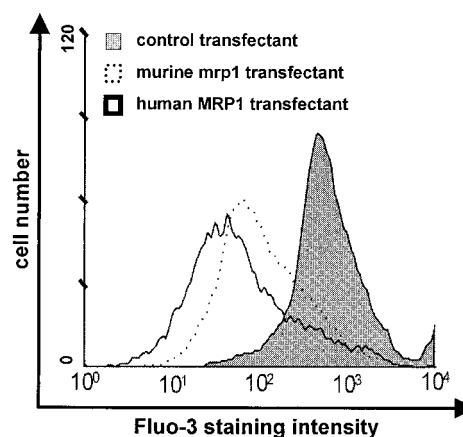
The Th1 cell clones used were B10BI derived from a C57BL/6 mouse and specific for bovine insulin, and LNC-2 derived from a BALB/c mouse and specific for *Mycobacterium tuberculosis*-purified protein derivative (PPD) (20, 21). The Th2 cell clone used was L1/1, derived from a BALB/c mouse, with specificity for LmAg, a *L. major* Ag preparation (20). The in vitro propagation of these T cell clones has been described (21). Briefly,  $5 \times 10^5$  cells/well were restimulated every 4 wk in 12-well tissue culture plates (Costar, Cambridge, MA) with syngeneic irradiated (25 Gy) spleen cells ( $5 \times 10^6$ /well) and Ag in a total volume of 3 ml of Clicks' RPMI 1640 medium (Biochrom, Berlin, Germany) supplement as described (22). Forty-eight hours after restimulation, the contents of the wells were divided into two, and culture supernatant (SN, 4%) of the cell line X63Ag8-653/IL-2 (23) was added as a source of recombinant murine (rm) IL-2. Thereafter, rmIL-2 was added once per week. Cells were routinely used for the experiments 4 wk after their final antigenic restimulation and 2 wk after the final addition of rmIL-2.

For in vitro restimulation,  $5 \times 10^5$  T cells were cultured in 24-well plates (Nunc, Wiesbaden, Germany) either in the presence of Ag and  $2 \times 10^6$  syngeneic spleen cells or in the presence of rm cytokines, as detailed in the legends of Figs. 1–9. After 48 h, the T cells were harvested and processed for the analysis of Fluo-3 extrusion and mrp1 expression. Cytokine production in the SNs of the stimulated T cells was determined in triplicate using commercial ELISA kits (PharMingen).

### Flow cytometry

For the labeling of T cells with Fluo-3-AM and secretion of the Fluo-3 anion, a procedure was used that is described in detail elsewhere (16). However, in the present study dye extrusion during labeling of cells with Fluo-3-AM in HBSS containing 5% FCS was inhibited by the presence of CsA (25  $\mu$ mol) instead of probenecid. After washing in HBSS/5% FCS, the cells were incubated for 10 min in the presence of CsA at 37°C to allow for complete export of the fluorogenic anion Fluo-3 after cleavage cytoplasmic esterases. Thereafter, CsA was washed out of the medium, and the cells were allowed to export Fluo-3 for 60 min at 37°C. Fluo-3 accumulation in the cells was analyzed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and the CellQuest software (Becton Dickinson). For determination of Fluo-3 extrusion in mrp1-transfected HEK 293 cells, a simplified procedure was followed. Thus, cells were labeled for 60 min with Fluo-3-AM in the absence of CsA to permit simultaneous uptake of Fluo-3-AM and secretion of Fluo-3. Steady-state Fluo-3 levels were determined immediately afterward.

For detection of an intracellular epitope of murine mrp1 at the single-cell level,  $1 \times 10^6$  cells were washed twice with PBS and incubated in PBS containing 2% paraformaldehyde at room temperature for 20 min. Cells were then permeabilized using 0.1% Triton-X-100 and 0.1% sodium citrate in H<sub>2</sub>O at 37°C for 10 min. After washing, the cells were incubated with



**FIGURE 1.** Human MRP1 and murine mrp1 transport the fluorescent anion Fluo-3. HEK 293 cells were transfected with the vector pcEBV7 containing either *MRP1* cDNA or *mrp1* cDNA. Control cells were transfected with the parental vector. The cells were incubated with Fluo-3-AM for 60 min at 37°C, and the Fluo-3 staining intensity was analyzed by flow cytometry. Two independent experiments were performed with similar results.

mAb MRP1 (1  $\mu$ g/ml) (24) in PBS containing 0.5% saponin and 2% FCS. In parallel, cells were labeled with a rat IgG2a isotype control Ab (PharMingen). Binding of mAb was revealed with PE-conjugated goat anti-rat (10  $\mu$ g/ml; Jackson ImmunoResearch, West Grove, PA) or indodicarbocyanine-conjugated donkey anti-rat Ig (1  $\mu$ g/ml; Jackson ImmunoResearch). Staining intensity of mrp1 was measured by flow cytometry.

In lymph node cells from SEB-injected mice, mrp1 was similarly labeled, and, in addition, the cells were stained with anti-CD4 mAb conjugated to Cy-Chrome (2  $\mu$ g/ml; PharMingen) and either anti-V $\beta$ 8 mAb (10  $\mu$ g/ml; PharMingen) or anti-V $\beta$ 4 mAb (1:50; BioSource International, Camarillo, CA), both conjugated to FITC.

For detection of the surface Ag CD69 after antigenic or cytokine stimulation, T cells were incubated with biotinylated rat anti-mouse CD69 mAb (3  $\mu$ g/ml; PharMingen) in PBS containing 1% FCS at 4°C for 10 min. Binding of mAb was visualized by streptavidin conjugated to PE (1  $\mu$ g/ml; PharMingen). Whenever T cells were stimulated with Ag and APCs, flow cytometry analysis allowed for a clear distinction and gating of the respective T cell population from the APCs on the basis of forward-site scatter characteristics. All data shown are gated on the T cell population.

### Histochemistry

For histochemistry, T cells were prepared and labeled as described above for mrp1 detection by flow cytometry. In an additional step, the fixed and labeled T cells were spun onto glass slides using the Cytospin 2 (Shandon, Frankfurt, Germany). Thereafter, the T cells were scanned and analyzed using the Leica TCS confocal microscope system (Leica, Heidelberg, Germany).

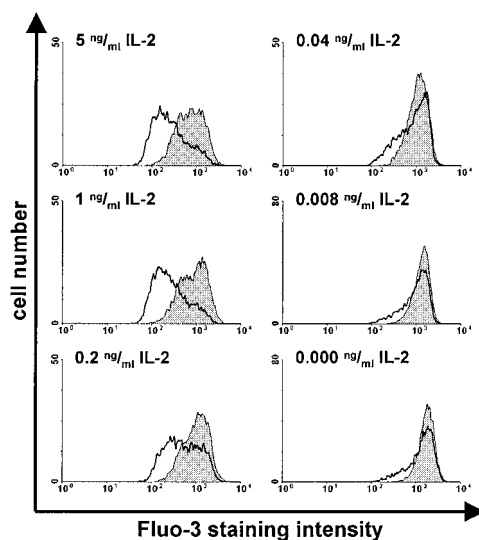
### Transfection of MRP cDNA

HEK 293 cells (25) were transfected with the episomal expression vector pcEBV7 (26, 27) containing murine mrp1 or human MRP1 cDNA. Control HEK 293 cells were transfected with the parental vector and grown under identical conditions, which are described in detail elsewhere (28).

## Results

### Transfection of mrp1 cDNA enables formerly inactive cells to transport the fluorescent anion Fluo-3

Previous analysis had established the existence of a protein pump in T cells that extrudes the fluorescent anionic dye Fluo-3 (16). Dye extrusion inhibition studies raised the possibility that the Fluo-3 anion pump was mrp1 (15). In experiments described here, we attempted to formally prove that mrp1 transports the Fluo-3 anion using HEK 293 cells transfected with the episomal expression vector pcEBV7 containing either the human *MRP1* or the



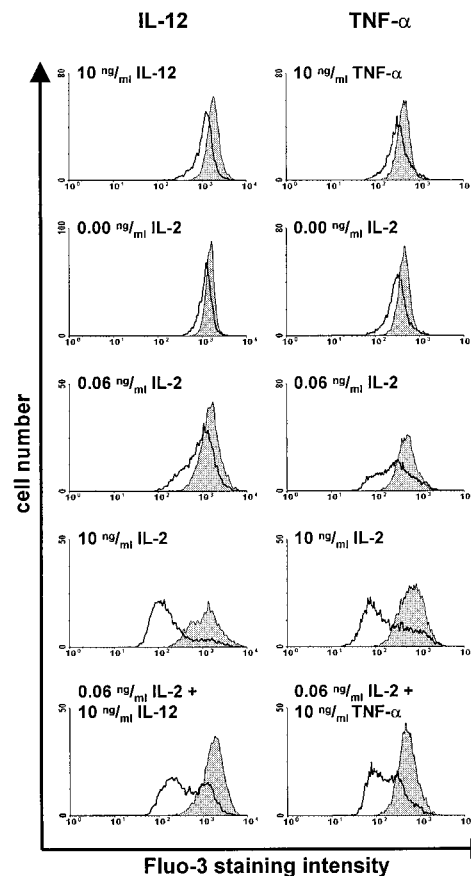
**FIGURE 2.** Fluo-3 extrusion is dose-dependently enhanced by IL-2. Resting Th1 cells (LNC-2) were stimulated with the indicated concentrations of murine rIL-2. After 48 h, T cells were labeled with Fluo-3-AM as described in *Materials and Methods*. After removal of CsA (25  $\mu$ mol), one-half of the cells were kept at 4°C (*shaded histograms*) and the other half was allowed to export Fluo-3 for 60 min at 37°C (*open histograms*) before flow cytometry analysis was performed. The results are representative of four independent experiments.

murine *mrp1* cDNA (28). The cells were incubated with the non-fluorescent hydrophobic acetomethyl ester Fluo-3-AM, and accumulation of the cleaved fluorescent anion Fluo-3 was allowed to reach steady state. After 60 min, the Fluo-3 staining intensity indicating the amount of intracellular Fluo-3 anion was analyzed (Fig. 1). Cells transfected with the human or murine MRP1 cDNA were considerably less stained, demonstrating enhanced Fluo-3 transport activity, as compared with control transfected cells. Thus, this experiment demonstrates that Fluo-3 is a substrate for murine *mrp1* and human MRP1 and can be used to measure transport function of these proteins.

#### Up-regulation of the Fluo-3 pump after cytokine restimulation

In our previous studies (15), Fluo-3-extruding activity was found to be enhanced in Th1 cells after *in vitro* restimulation with Ag and APCs. In this study, we show that, in addition, Fluo-3 extrusion is up-regulated in Th1 cells after stimulation by cytokines. Th1 cells of the two clones B10BI and LNC-2 were incubated with different concentrations of IL-2. After 48 h, T cells were harvested and the Fluo-3 extrusion capacity was tested (Fig. 2). Comparison of the degree of Fluo-3 staining intensity immediately after labeling in the presence of CsA (Fig. 2, *shaded histograms*) and after 60 min of incubation in the absence of CsA (Fig. 2, *open histograms*) shows that IL-2 dose-dependently up-regulated the Fluo-3-extruding activity. Dye extrusion could be inhibited by further incubation in CsA (data not shown). The data obtained for the two clones were comparable and are given for only one of them.

IL-12 and TNF- $\alpha$ , which by themselves were almost unable to induce Fluo-3 extrusion, were very potent Fluo-3 pump activators when combined with a limiting concentration of IL-2 (Fig. 3). In these experiments, the combination of rIL-12 (10 ng/ml; Fig. 3, *left panel*) or of rTNF- $\alpha$  (10 ng/ml; Fig. 3, *right panel*) with a low concentration of rIL-2 (0.06 ng/ml) induced the Fluo-3 pump activity to the same level as the optimal amount of rIL-2 (10 ng/ml) alone. Similar data were obtained for the Th1 cell clones B10BI (Fig. 3) and LNC-2 (data not shown). In contrast, the cytokines IFN- $\gamma$ ,



**FIGURE 3.** Synergistic effects of cytokine combinations on Fluo-3 extrusion in Th1 cells. Resting Th1 cells (B10BI) were restimulated with the cytokines rIL-2 and either rIL-12 (*left panel*) or TNF- $\alpha$  (*right panel*) as indicated. After 48 h, cells were labeled with Fluo-3-AM and tested for Fluo-3 extrusion as described in Fig. 2. The results are representative of three independent experiments.

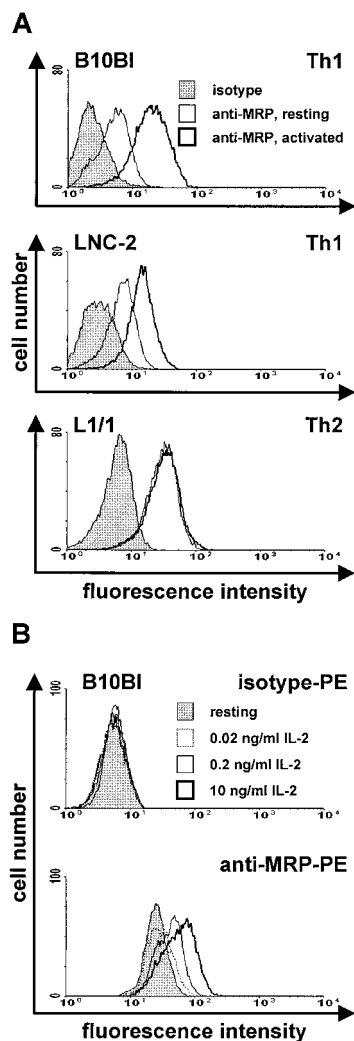
IFN- $\gamma$ , GM-CSF, IL-1 $\beta$ , IL-3, IL-4, IL-6, IL-9, IL-10, and IL-15 were unable to induce any Fluo-3 pump activity (data not shown).

#### Detection of *mrp1* in CD4<sup>+</sup> T cells at the single-cell level

Next, we analyzed, whether the up-regulation of Fluo-3 extrusion activity, was accompanied by an increase in *mrp1* protein at the single-cell level. Resting Th1 and Th2 cells were stimulated with optimal amounts of rIL-2 (10 ng/ml) for 48 h (Fig. 4A). Thereafter, the clones were processed for fluorescence staining of an intracellular *mrp1* epitope with mAb MRPr1. Resting Th1 cells displayed a weak *mrp1* signal compared with resting Th2 cells, confirming our earlier observations (15). After cytokine stimulation, *mrp1* expression was unchanged in Th2 cells, but was elevated in Th1 cells to almost the same level as in Th2 cells. Moreover, *mrp1* expression in Th1 cells was up-regulated by IL-2 dose dependently (Fig. 4B), confirming the above reported effects of IL-2 on *mrp1* function (Fig. 2).

The ability to detect MRP at the single-cell level permitted the localization of the *mrp1* protein in the T cells by confocal laser microscopy. Again, *mrp1* could be detected only in resting Th2 cells and IL-2-activated Th1 cells but not in resting Th1 cells. In all cells expressing *mrp1*, i.e., in resting Th2 cells and activated Th1 cells, *mrp1* was localized almost exclusively at the plasma membrane (Fig. 5). No staining was observed with an isotype control mAb (Fig. 5).





**FIGURE 4.** Expression of mrp1 is enhanced by exposure of cells to IL-2. Resting Th1 and Th2 cells were restimulated with rmIL-2 for 48 h (A, 10 ng/ml; B, as indicated). For detection of mrp1 at the single-cell level, cells were fixed, permeabilized, and labeled with mAb MRPr1 or an isotype control. Ab binding was detected with indodicarbocyanine-conjugated donkey anti-rat Ab (A) or PE-conjugated goat anti-rat Ab (B). The results are representative of six independent experiments.

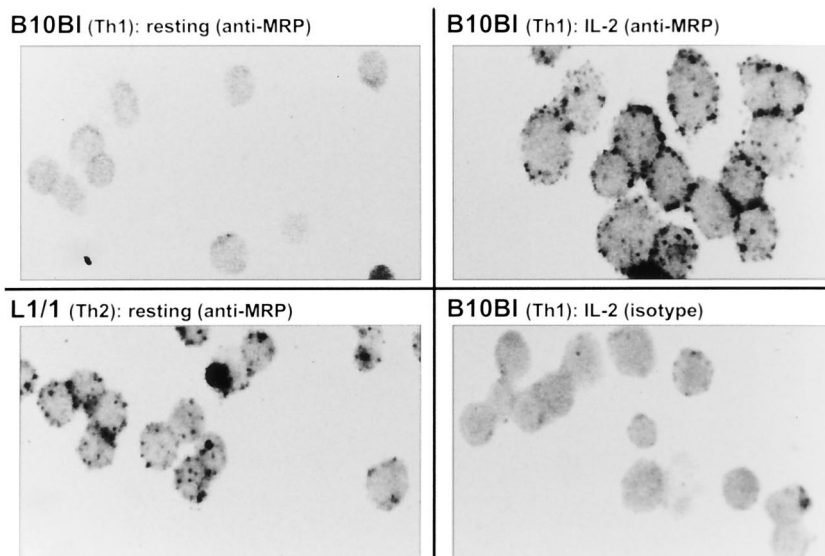
#### *In vivo mrp1 induction in CD4<sup>+</sup> lymphocytes by superantigen*

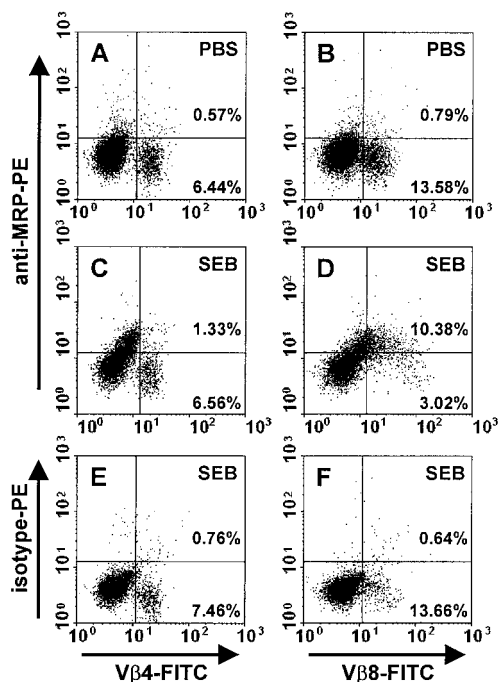
So far, our studies have demonstrated that mrp1 is an activation marker for long-term in vitro cultured Th1 cell clones. To verify that our findings also apply for CD4<sup>+</sup> T cells in vivo, we injected the superantigen SEB into the hind footpad of BALB/c mice. SEB activates T cells expressing the V $\beta$ 8 family of the TCR (29, 30). This experimental setup allowed us to study mrp1 expression in TCR-triggered V $\beta$ 8<sup>+</sup> T cells compared with V $\beta$ 4<sup>+</sup> T cells, as a negative control. Twelve hours after injection, the popliteal lymph node cells from SEB-injected and from vehicle-injected control mice were processed for a three-color staining of mrp1, CD4, and V $\beta$ 4 or V $\beta$ 8. The panels in Fig. 6 depict lymphocytes gated for CD4<sup>+</sup> expression. Control mice (Fig. 6, A and B) demonstrated almost no mrp1 expression in the V $\beta$ 4<sup>+</sup> or V $\beta$ 8<sup>+</sup> cells. In SEB-injected mice, mrp1 expression in the irrelevant V $\beta$ 4<sup>+</sup> TCR subset was comparable to that of control mice (Fig. 6C, upper right quadrant), whereas the relevant V $\beta$ 8<sup>+</sup> TCR subset was almost entirely mrp1 positive (Fig. 6D, upper right quadrant). The negative result of the isotype control labeling in the SEB-injected mice (Fig. 6, E and F) confirms the specificity of the mrp1 detection by mAb MRPr1. In a normal mouse, the V $\beta$ 8<sup>+</sup> TCR subset consists mostly of naive T cells; consequently, our findings demonstrate the in vivo reliability of mrp1 as a marker for T lymphocytes that have been triggered for the first time and only for 12 h via their TCR.

#### *The specific MRP inhibitor MK571 suppresses Th cell activation*

In another set of experiments, we examined the physiological relevance of mrp1 in the activation process of Th1 and Th2 cells. Resting Th1 cells were stimulated for 48 h with rmIL-2 (Fig. 7, A–C) or Ag and APCs (Fig. 7, D–F) in the presence or absence of MK571, an inhibitor of MRP (31). A shift in the forward scatter confirmed the activation of the Th1 cells by both stimuli. Importantly, this shift was reduced to the value of resting Th1 cells when MK571 (75  $\mu$ M) was added (Fig. 7, A and D). Along with blastogenesis, expression of the activation marker CD69 (32) was increased by IL-2 or antigenic stimulation. Like blastogenesis, CD69 expression was markedly blocked by simultaneously added MK571 (75  $\mu$ M) (Fig. 7, B and E). As a third parameter for Th1 cell activation, the production of IFN- $\gamma$  was analyzed. In the absence of MK571, both Th1 clones produced IFN- $\gamma$  in response to IL-2 (Fig. 7C) or Ag plus APCs (Fig. 7F). In the presence of MK571, the secretion of IFN- $\gamma$  was blocked. Similar results were

**FIGURE 5.** The mrp1 protein is located at the plasma membrane of resting Th2 cells and activated Th1 cells. Resting B10BI (Th1) cells were stimulated with rmIL-2 for 48 h. For detection of mrp1 protein, cells were processed as described in Fig. 4. Using PE-conjugated goat anti-rat Ab, the binding of the mAb MRPr1 or the isotype control was detected. After labeling, cells were spun onto glass slides and prepared for confocal laser microscopy. The results are representative of six independent experiments.





**FIGURE 6.** Mice injected with SEB show enhanced mrp1 levels only in T cells expressing the SEB-reactive Vβ8<sup>+</sup> family. Female BALB/c mice were injected with 10 μg of SEB per 50 μl PBS into the hind footpad. Control mice received PBS only. After 12 h, the draining popliteal lymph nodes were removed. The lymph node cells were processed for mrp1 detection as described in Fig. 4. In addition, the cells were stained with anti-CD4 Cy-Chrome and either anti-Vβ8 FITC (B, D, and F) or anti-Vβ4 FITC (A, C, and E). All panels show gated CD4<sup>+</sup> lymph node cells. Two independent experiments were performed with similar results.

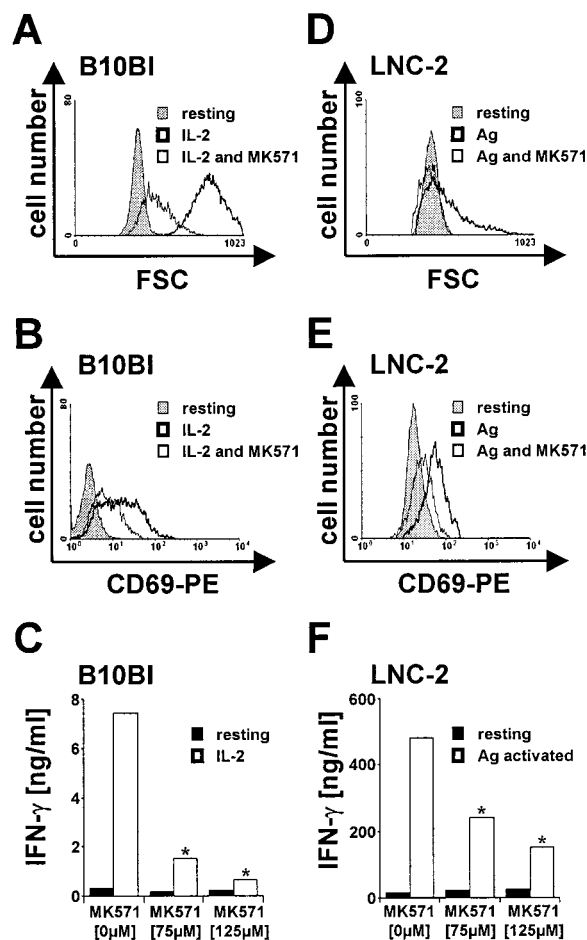
obtained when the Th1 cells were cocultured with PSC 833, a different MRP inhibitor (15), instead of MK571 (data not shown). In addition, the effect of MK571 was not restricted to Th1 cells in that the Ag-induced secretion of IL-4 by Th2 cells was also distinctly blocked by simultaneous incubation with MK571 (Fig. 8B). In a direct comparison, the MK571 effect on Th2 cells tended to be smaller than the one on Th1 cells (Fig. 8, A and B).

In control experiments, toxic effects of MK571 on activated Th cells could be excluded (Fig. 9). To show this, resting Th1 cells were stimulated with rmIL-2 for 24 h, leading to a shift in CD69 expression (Fig. 9A). Thereafter, the cells were washed and further incubated either in medium only or in medium containing the MRP-specific inhibitor MK571. After an additional 6- or 12-h incubation, CD69 expression had not changed in the MK571 group compared with the control group (Fig. 9B). Likewise, IFN-γ production was not reduced after 6 or 12 h in the culture SNs containing MK571 compared with the control samples.

In conclusion, inhibition of MRP limits the Th1 cell response to activation via two different stimuli such as cytokines or Ag plus APCs. To be effective, however, inhibition has to occur during the initiation of T cell activation and not once the T cells are already activated.

## Discussion

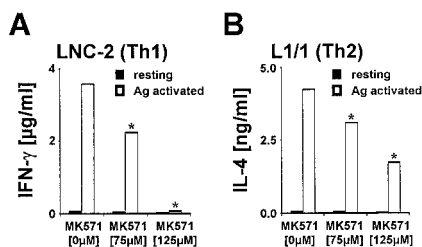
In this study, we demonstrate that the fluorescent anion Fluo-3 can be transported by the human MRP1, a known mediator of multidrug resistance in tumor cells and its murine ortholog mrp1. Although this is not unexpected given the known substrate specificity of MRP1/mrp1, this is the first direct demonstration that Fluo-3



**FIGURE 7.** The specific MRP inhibitor MK571 suppresses Th1 cell activation in vitro. Resting B10BI Th1 cells were stimulated for 48 h with rmIL-2 (10 ng/ml) in the presence or absence of MK571 (75 μM in A and B and as indicated in C). Resting LNC-2 cells (Th1 cell line) were activated for 48 h with Ag (PPD) and APCs. The cells were cultured in the presence or absence of MK571 (75 μM in D and E, and as indicated in F). Thereafter, the size (A and D) or the level of CD69 expression (B and E) was measured by flow cytometry, and IFN-γ production (C and F) was tested in 48-h SN by ELISA. The results are representative of three independent experiments. Bars depict the median. \*,  $p < 0.05$  with respect to the sample without MK571; Mann-Whitney  $U$  test.

can be used to monitor MRP1/mrp1 activity. Identification of MRP1/mrp1-positive cells by Fluo-3-labeling instead of Ab-labeling has the significant advantage that the cells to be tested remain viable, can be isolated using flow cytometry, and can be used for further functional tests or long-term in vitro cultures. So far, the only mAbs available for MRP1/mrp1 detection recognize intracellular epitopes and therefore require fixation and subsequent permeabilization of the cells (24, 33).

We have recently hypothesized that mrp1 is present and functional in nontumorigenic, cloned murine Th cells (15). This hypothesis was based on dye extrusion inhibition studies as well as on a strong correlation between the presence of the Fluo-3 pump and the expression of the *mrp1* gene. The data presented here based on studies with MRP1/mrp1-transfected cells corroborate our original hypothesis. We have also previously shown that the Fluo-3 pump was differentially expressed in Th cells in that resting Th2 cells, but not resting Th1 cells, exported the Fluo-3 anion. After restimulation with Ag and APCs, Th1 cells acquired the potential to extrude Fluo-3 to the same extent as resting Th2 cells.



**FIGURE 8.** The specific MRP inhibitor MK571 suppresses not only Th1, but also Th2 cell activation in vitro. Resting LNC-2 Th1 and L1/1 Th2 cells were stimulated for 48 h with specific Ag (LNC-2, PPD; L1/1, LmAg) and syngenic splenic APCs in the presence or absence of MK571. Production of IFN- $\gamma$  (A) and IL-4 (B) was tested in 48-h SN by ELISA. Two independent experiments were performed with similar results. Bars depict the median. \*,  $p < 0.05$  with respect to the sample without MK571; Mann-Whitney  $U$  test.

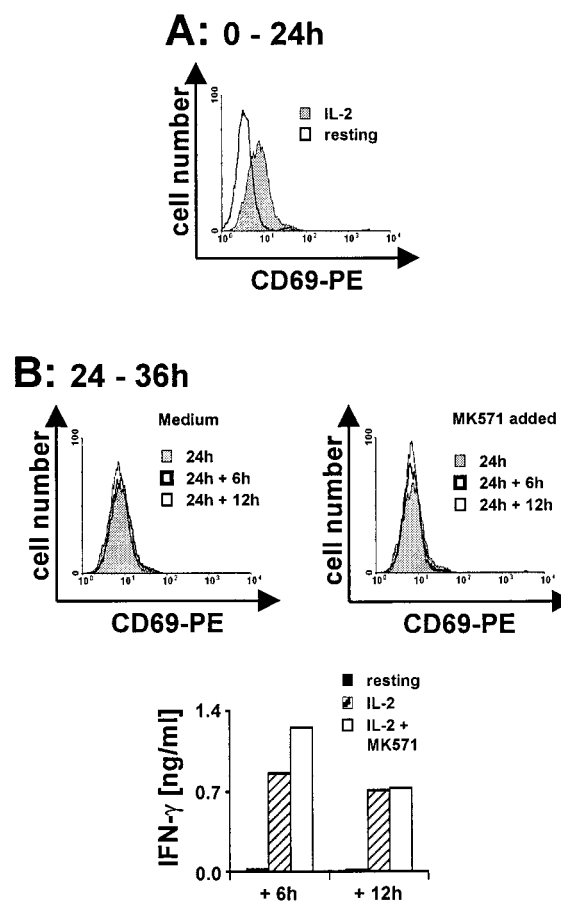
This was revealed at the level of Th1 and Th2 cell clones in vitro as well as at the level of freshly purified Th1 and Th2 cells ex vivo. There was no change of the Fluo-3 pump activity in Th2 cells after antigenic restimulation compared with the resting state.

We now report that in addition to antigenic restimulation, several cytokines are able to up-regulate the expression of the Fluo-3 pump in Th1 cells. IL-2 seems to be the most powerful cytokine in this regard. IL-12 and TNF- $\alpha$  were substantially less effective than IL-2, but both cytokines strongly synergized with a limiting concentration of IL-2. It should be mentioned that we were unable to influence the Ag-triggered induction of the Fluo-3 pump with a neutralizing anti-IL-2 Ab (data not shown). We are currently pursuing the hypothesis that a TCR signal may up-regulate the Fluo-3 pump independently of cytokines.

Again, not only the Fluo-3 pump activity was up-regulated after IL-2 stimulation, but also mrp1 protein expression, as revealed by FACS analysis at the single-cell level. Therefore, Fluo-3 pump up-regulation correlated with up-regulation of mrp1 protein, providing further evidence that the Fluo-3 pump is mrp1. Together, our studies clearly implicate mrp1 as an activation marker for Th1 cells in vitro. In addition, mrp1 is constitutively expressed in Th2 cells, at least at the level of T cell clones.

The present study also demonstrates that mrp1 is an activation marker for Th cells in vivo. We had already suspected that this was the case on the basis of the Fluo-3 pump levels expressed during experimental murine leishmaniasis (15). Almost all *L. major* Ag-specific CD4<sup>+</sup> T cells were detected within the Fluo-3-extruding cell subset. Here, we extend these earlier findings and report that indeed mrp1 protein is specifically induced in CD4<sup>+</sup> T cells in vivo after TCR stimulation. Unfortunately, permeabilization of the cells needed for detection of intracellular mrp1 epitopes leads to diffusion of the Fluo-3 dye. This prevented double staining of mrp1 protein and the activity of the Fluo-3 pump in *L. major* Ag-specific T cells and prevented the use of the *Leishmania* model, in which it is impossible to directly identify the *L. major*-specific CD4<sup>+</sup> T cells. Therefore, we used the model Ag SEB, which binds to MHC II molecules, and in this state directly interacts with any TCR containing a  $\beta$ -chain of the V $\beta$ 8 family. Therefore, all V $\beta$ 8<sup>+</sup> T cells, which can be identified by Ab staining, should be recognized and activated by SEB (29). We found that already 12 h after SEB injection into mice, only the relevant TCR V $\beta$ 8 subset, but not the V $\beta$ 4 subset used as a control, contained elevated levels of the mrp1 protein.

Taken together, we propose mrp1 as a new activation marker for murine T cells, not only in long-term in vitro cultures but also in freshly ex vivo-isolated murine T cells. To our knowledge, this is



**FIGURE 9.** MK571 has no toxic effects on activated Th1 cells. Resting Th1 cells were activated with IL-2 (10 ng/ml) for 24 h and CD69 expression was documented by FACS (A). After 24 h, the cells were washed and further incubated in the presence or absence of the MRP-specific inhibitor MK571 (75  $\mu$ M) for the indicated time period. Thereafter, CD69 expression was measured by FACS (B), and the SNs were tested for IFN- $\gamma$  production. Two independent experiments were performed with similar results. Bars depict the median.

the first report that characterizes mrp1 as an inducible molecule in untransformed cells rather than tumor cells. So far it was only known that low levels of MRP mRNA are present in normal peripheral blood CD4<sup>+</sup> T cells (19).

The role of mrp1 in the activation process of Th cells is not known. MRP knockout mice had increased levels of glutathione in blood mononuclear leukocytes as well as in bone marrow cells and erythrocytes, indicating that mrp1 might pump this reducing tripeptide (34). Glutathione conjugates such as leukotriene C<sub>4</sub> (LTC<sub>4</sub>) have been demonstrated to be transported by MRP1 in vitro (31, 35, 36). LTC<sub>4</sub> is involved in a number of signal transduction pathways that control vascular permeability and smooth muscle contraction (37). Not surprisingly, therefore, mrp1-deficient mice displayed an impaired response to an inflammatory stimulus as a result of their defective LTC<sub>4</sub> secretion (38). In vitro studies have also shown that oxidized glutathione acts as a substrate for MRP (39). Consequently, it has been suggested that MRP may be involved in protection against oxidative stress and maintenance of the intracellular redox potential (40). In T cells, IL-2 initiates proliferation which results in enhanced metabolic activity, accumulation of oxygen intermediates, and changes in the intracellular redox potential. Our results raise the possibility that one of the responses to this change in redox state is up-regulation of mrp1



expression. On the other hand, IL-12 triggers Th1 differentiation (41). Therefore, the combination of IL-2 and IL-12 triggers two different signal transduction pathways for proliferation and differentiation and leads to strongly enhanced metabolism. This could explain the necessity for synergistic expression of mrp1 in Th1 cells stimulated by IL-2 plus IL-12. TNF- $\alpha$ , which we have shown to synergize with IL-2, has been reported previously to increase MRP expression at the level of mRNA and protein in a colon carcinoma cell line (42).

Alternatively, it is conceivable that mrp1 is directly involved in cytokine secretion. It is known that P-glycoprotein, another member of the multidrug resistance group of proteins, is involved in the transport of IL-2 in PHA-stimulated human peripheral blood leukocytes (43). However, this transporter is not expressed in the herein used murine Th1 clones (15) and its role may be filled by mrp1.

Whatever the role of mrp1 in murine T cells may be, it has to account for the fact that this molecule is constitutively expressed in Th2 cells (15). The significance of this finding for the physiology of Th2 cells is completely unknown and under investigation. Possibly, Th2 cells have an increased level of cell metabolism even in the resting state, which requires that the detoxification machinery is continuously present.

One would expect that for mrp1 to be functional in protection against oxidative stress or in cytokine secretion, the protein should be localized in the plasma membrane. Using confocal laser microscopy, we have confirmed that this is the case in both resting Th2 as well as stimulated Th1 cells. Previous studies examined MRP localization in MRP-overexpressing tumor cells and MRP gene-transfected cells and came to similar conclusions (44, 45).

The idea of MRP as a quick detoxifier of the cell may also explain a discrepant result for Fluo-3 extrusion and mrp1 protein expression in resting Th1 cells. Here, a significant baseline expression of mrp1 protein was detectable by fluorescence staining, whereas no Fluo-3 extrusion occurred. At present, we are unable to exclude that this baseline staining reflects nonspecific binding of the mAb MRPr1. Alternatively, however, the reason for this discrepancy may reside in the fact that the MRP molecule has two nucleotide binding sites (46), both of which have to be occupied by ATP molecules for functionality. Thus, inactive mrp1 may be located at the membrane (and detectable by Ab staining) to keep the cell alert for a critical situation when quick detoxification or cytokine secretion is needed. Such a scenario would enable the cell to react much faster toward changes in the micromilieu, as it could, if de novo mrp1 synthesis was required.

In an attempt to determine whether the elevated expression of mrp1 in activation of Th1 cells has a functional role in the activation process, we stimulated Th1 cells in the presence of the MRP-specific inhibitor MK571 (31). Blastogenesis as well as CD69 (32) expression was reduced in Th1 cells coincubated with MK571. Thus, blocking the mrp1 transport during the initiation of Th1 activation appears to interfere with completion of the activation process. Similarly, IFN- $\gamma$  production was inhibited by MK571 to the level of resting cells. Similar results were obtained when MRP was blocked by PSC 833 instead of MK571, indicating that the effects of MK571 were not restricted to this compound. In Th2 cells, MK571 interfered with the Ag-induced secretion of IL-4. This set of data indicates that mrp1 transport is essential not only for Th1, but also for Th2 cell-mediated immunity.

MK571 works at the inner leaflet of the plasma membrane, and therefore published data on inhibition of MRP by MK571 used inside-out vesicles and relatively low amounts of the inhibitor (31). In our studies, we tested the effects of MK571 on viable T cells, which required higher concentrations of the inhibitor. To exclude

artificial results caused by a putative toxicity of MK571 at the concentrations used, we incubated already activated T cells with MK571 for an additional 12 h. No changes in IFN- $\gamma$  production, blastogenesis (data not shown) and CD69 expression were observed compared with the non-MK571-treated control. Therefore, a toxic effect of MK571 on T cell function could be excluded. Thus, these results clearly demonstrate that MRP inhibition only influences T cell function when it occurs along with the initiation of T cell activation, but not once T cells are already activated.

Taken together, these data demonstrate that mrp1 function is necessary for efficient Th1 cell activation, suggesting that the protein either secretes molecules such as cytokines that are involved in activation or effluxes intracellular inhibitors of the process. Further studies will try to identify intracellular signaling pathways that are blocked by MK571 and may shed light on the exact molecular nature of MRP function in this context.

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