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Relation between mRNA Expression Level of MDR1/ABCB1 in Blood Cells and Required Level of Tacrolimus in Pediatric Living-donor Liver Transplantation

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Running Title: Blood MDR1 on tacrolimus target level after liver transplant

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Abbreviations: MDR, multidrug resistance; ACR, acute cellular rejection; LDLT, living-donor liver transplantation; PBMC, peripheral blood mononuclear cell

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Abstract

It has been difficult to set an individualized therapeutic window of tacrolimus after organ transplantation, because of wide interindividual variation of responsiveness to immunosuppressive therapy. In this study, we examined the significance of multidrug resistance 1 (MDR1) in the peripheral blood cells by comparing the trough concentration of tacrolimus with the occurrence of acute cellular rejection (ACR) in retrospectively collected pediatric living-donor liver transplant (LDLT) patients, who were enrolled after obtaining written informed consent. No significant difference in the intraindividual variation in MDR1 mRNA expression in the peripheral blood cells was observed between postoperative days 3 and 7. The average trough concentration of tacrolimus during the 15-day postoperative period was significantly higher in the event-free patients than in those who experienced ACR (21 of 44 cases) and had higher levels of blood MDR1 mRNA. In addition, the average trough concentration of tacrolimus significantly correlated with the logarithmically transformed MDR1 mRNA data from the blood cells in patients of both the event-free ($r=0.5406$, $P=0.0077$) and ACR ($r=0.4772$, $P=0.0284$). The cellular accumulation of [^{14}C]tacrolimus in the peripheral blood mononuclear cells was 2-fold higher in *mdr1a/1b*-knockout mice than in wild-type mice ($P=0.0182$). These results suggest that MDR1 in blood cells decreases the leukocytic concentration of tacrolimus and could be a useful marker to establish an individualized target concentration of tacrolimus to prevent ACR in pediatric patients after liver transplantation.

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Introduction

Tacrolimus, a calcineurin inhibitor, is widely used as an immunosuppressant in patients undergoing organ transplantation. It acts on T lymphocytes and inhibits the NFAT-derived production of interleukin 2 and the subsequent proliferation of lymphocytes, thereby contributing to a marked improvement in graft survival (Denton et al., 1999; Scott et al., 2003). The therapeutic range of tacrolimus for liver transplantation is considered to be approximately 5–20 ng/mL, but the blood concentration of tacrolimus during the development of acute cellular rejection (ACR) differs among patients (Yasuhara et al., 1995). Because no sensitive marker of drug effectiveness has yet been identified, the establishment of a dosage regimen based on individual susceptibility is required.

P-glycoprotein is the product of multidrug resistance gene *MDR1/ABCB1*. It exists in several tissues such as the intestine, liver, and kidney and prevents the intracellular accumulation of numerous drugs (Cordon-Cardo et al., 1990; Hoffmann and Kroemer, 2004). P-glycoprotein in the intestine and kidney is considered to influence the pharmacokinetics of various types of drugs during absorption and tubular excretion, and some drug interactions via the transporter have also been reported (Wakasugi et al., 1998; Greiner et al., 1999). It is also expressed in various types of blood cells; depending on its expression level, it is reported to prevent the uptake of doxorubicin (Klimecki et al., 1994). Further, it has also been reported as a prognostic factor to predict relapse of childhood acute lymphoblastic leukemia (Dhooge et al., 1999). Based on these findings, it is hypothesized that P-glycoprotein in the peripheral blood cells also decreases the leukocytic concentration of tacrolimus,

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and its expression level is related to the frequency of ACR in liver transplant patients.

In the present study, we examined the relationship among the MDR1 mRNA expression level in the peripheral blood cells, tacrolimus trough concentration, and occurrence of ACR in pediatric patients undergoing living-donor liver transplantation (LDLT). Further, we compared the intracellular concentration of tacrolimus in the peripheral blood mononuclear cells (PBMCs) between *mdr1a/1b* null mice and wild-type mice.

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Methods

Patients, dosage regimen of tacrolimus, analysis of blood samples, and criteria for ACR

Between October 2001 and September 2003, 44 pediatric patients who underwent LDLT in Kyoto University Hospital were enrolled in this study after written informed consent was obtained from them and/or their parents. The patients were aged between 0.25 and 14 years. The primary diseases were biliary atresia (33 patients, including 1 patient with a liver tumor and 1 with Alagille syndrome), Alagille syndrome (2 patients), hyperpropionemia (2 patients), Wilson disease (1 patient), primary sclerosing cholangitis (1 patient), Byler disease (1 patient), and re-LDLT (4 patients). The blood type matches between the donors and recipients were identical in 34 cases and compatible in 10 cases. The demographics of the control event-free group and the ACR group are shown in Table 1. This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Kyoto University.

After transplantation, immunosuppression by oral administration of tacrolimus (0.04 mg/kg) every 12 h was initiated at 12 h after reperfusion (Asonuma et al., 1998). The target trough blood concentration of tacrolimus was 10–12 ng/mL during the first 2 weeks after transplantation. The daily oral dose was modulated based on the whole-blood tacrolimus concentration that was measured approximately 12 h after the evening administration by using a semiautomated microparticle enzyme immunoassay (IMx®, Abbott, Tokyo, Japan) (Yasuhara et al., 1995).

ACR was defined by the biochemical abnormalities that were mainly

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evaluated by the re-elevation of transaminases and by the histological evaluation of liver biopsy specimens. The patients who were diagnosed with ACR were treated with a high-dose intravenous administration of methylprednisolone or corticosterone.

Evaluation of MDR1 mRNA expression level in blood

The blood samples for the examination of mRNA expression were collected in a PAXgene Blood RNA Tube (Qiagen Gmbh, Hilden, Germany) on postoperative days 3 and 7. The total RNA was extracted using the PAXgene Blood RNA Kit (Qiagen) and reverse transcribed by SuperScript® II transcriptase (Invitrogen, Carlsbad, CA) after the digestion of contaminated genomic DNA by RQ1 DNase (Promega Co., Tokyo, Japan), as previously described (Masuda et al., 2000). The mRNA expression level of MDR1 was quantitated by real-time PCR using the Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The primer and TaqMan probe sets used have been described previously (Uwai et al., 2004).

Distribution of [^{14}C]tacrolimus in PBMCs in mdr1a/1b null mice

FVB control wild-type mice and *mdr1a/1b* double knockout mice weighing 30–40 g (Taconic Farms Inc., Albany, NY) were used. Prior to the experiments, the mice were housed in a temperature- and humidity-controlled room and allowed free access to water and standard chow. The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University. The experimental protocol was approved by the Animal Research Committee, Graduate

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After diethylether anesthesia, whole blood sample was collected from the aorta, and biochemical parameters were confirmed using the I-STAT® analyzer (Abbott). The mean hematocrit values of the FVB control mice (n = 7) and *mdr1a/1b* null mice (n = 8) were 32.9% and 30.9%, respectively (P = 0.3315 by Student's *t* test). The blood levels of Na^+, K^+, Cl^-, blood urea nitrogen, and glucose (data not shown) did not exhibit abnormal values. The [^{14}C]tacrolimus (683 kBq/mg, kindly provided by Astellas Pharma Co., Tokyo, Japan) was spiked in the whole blood (6.4 kBq/mL, 10.4 µg/mL) and incubated at 37°C for 30 min with gentle shaking. At the end of the incubation, the whole blood was diluted with the same volume of phosphate-buffered saline (pH 7.4) to isolate PBMCs by using Ficoll-Paque Plus (GE Healthcare Bioscience, Tokyo, Japan) (Fukudo et al., 2005a; Fukudo et al., 2005b). The contaminating red blood cells were removed with red blood cell lysis buffer (Roche Diagnostics KK, Tokyo, Japan). The PBMC samples were dissolved using 0.5 mL of NCS tissue solubilizer (GE Healthcare Bioscience), and liquid scintillation counting was performed with 3 mL of the ACSII scintillation cocktail (GE Healthcare Bioscience).

Statistics

Statistical analysis was performed using Prism Version 4.0 software (GraphPad, San Diego, CA). Logarithmic transformation of the mRNA levels of MDR1 was performed to improve normality before performing statistical analyses such as distribution examination and correlation analysis. Statistical differences

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between the 2 groups were analyzed by Student's *t* test and the Mann–Whitney U test after performing analysis of normality and the F test.

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Results

MDR1 mRNA expression level in the peripheral blood cells

Figure 1A shows a histogram of logarithmically transformed data representing the expression levels of MDR1 mRNA in the peripheral blood cells at postoperative day 3. The median values of MDR1 mRNA in the all patients at postoperative days 3 and 7 were 0.05 and 0.09 amol/μg total RNA, respectively. The MDR1 mRNA level in the peripheral blood cells was compared between postoperative days 7 and 3 in each of the 32 patients (Fig. 1B). Because the high-dose steroid pulse therapy was administered between postoperative days 3 and 7 to 7 patients, their MDR1 mRNA expression data were excluded. The blood samples of 5 patients could not be obtained due to patient refusal. The distribution of the MDR1 mRNA level exhibited a similar unimodal pattern in both control cases (event-free, *empty column*) as well as in the ACR cases (*filled column*) (Figs. 1A and 1C); further, there was no statistically significant difference in the MDR1 mRNA levels between postoperative days 3 and 7 (data not shown), and in the fold changes of the MDR1 mRNA levels during postoperative days 3 and 7 between the control and ACR cases (Fig. 1B). Therefore, the molecular data at postoperative day 3 was used for further examinations.

Blood concentration profiles of tacrolimus, MDR1 mRNA level in blood cells, and ACR

The blood concentration profiles of tacrolimus during the 15-day postoperative period are shown in Fig. 2. The patients were categorized into the

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following 4 groups based on the mRNA expression level of MDR1 at postoperative day 3: below 0.02, between 0.02 and 0.05, between 0.05 and 0.1, and higher than 0.1 amol/μg total RNA. The open circles represent the trough concentrations of tacrolimus a day prior to the initiation of the increase in transaminase levels. The tacrolimus concentrations in 10 patients who experienced ACR were below 5 ng/mL immediately before the increase in transaminase levels. The patients who experienced ACR after postoperative day 10 exhibited a blood tacrolimus concentration profile that increased rapidly a few days after the operation and decreased by nearly 5 ng/mL preceding the occurrence of ACR. Although the trough concentrations of tacrolimus were higher than 10 ng/mL, episodes of ACR occurred in 3 patients.

In order to obtain additional information on the association of the MDR1 mRNA expression level in the peripheral blood cells with the tacrolimus blood concentration required to avoid ACR, the χ^2 test was carried out and the odds ratio was calculated. After step-by-step examinations, we determined the cut-off value of 9 ng/mL of the average tacrolimus blood concentration during 15 postoperative days for subsequent statistical analyses. As shown in Table 2, the significance of a minimum tacrolimus blood concentration of 9 ng/mL during the 15-day postoperative period was found in 44 patients in this study ($P = 0.0004$). Next, we examined the effect of the MDR1 mRNA expression level on the frequency of ACR after classifying the patients based on the median expression level (0.05 amol/μg total RNA). The importance of tacrolimus blood concentration was observed in patients with a higher as well as lower MDR1 mRNA expression level in blood cells (higher MDR1, n = 22,

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P = 0.0112; lower MDR1, n = 22, P = 0.01543). The odds ratio revealed that the average trough level of tacrolimus of <9 ng/mL was a significant risk factor for ACR, particularly in patients with a high level of MDR1 mRNA expression in the blood cells (Table 2).

Correlation between the MDR1 mRNA expression level in blood cells and the average trough levels of tacrolimus during the 15-day postoperative period

In order to examine whether the MDR1 mRNA expression level at postoperative day 3 was associated with the individual target trough concentration of tacrolimus during the early phase after liver transplantation, the relationship between the molecular data and the average trough concentration of tacrolimus during the 15-day postoperative period was investigated in the event-free and ACR patients. Because high-dose steroid injection treatment (pulse therapy) is a strong immunosuppressive treatment against ACR, the data of trough concentration of tacrolimus with regard to this treatment were excluded in the patients who experienced ACR. Although the data was logarithmically transformed, the observed blood MDR1 mRNA expression level was weak, but it significantly correlated with the average trough concentration of tacrolimus in both event-free patients (R = 0.5406, P = 0.0077, Fig. 3A) and ACR patients (R = 0.4772, P = 0.0284; Fig. 3B). The average trough level of tacrolimus was higher in the event-free patients than in the ACR patients (P = 0.0008; Fig. 3C).

Comparison of [^{14}C]tacrolimus accumulation in the PBMCs of mdr1a/1b knockout

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mice and wild-type mice

The pharmacological significance of blood MDR1 on the cellular accumulation of tacrolimus was investigated using the PBMCs derived from wild-type or *mdr1a/1b* knockout mice. After the incubation of whole blood samples with [^{14}C]tacrolimus, its accumulation in the PBMCs was examined. As shown in Fig. 4, the [^{14}C]tacrolimus concentration in the PBMCs of the *mdr1a/1b* knockout mice was 2-fold higher than that in the PBMCs of the wild-type mice ($P = 0.0182$).

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Discussion

MDR1 is expressed in various types of PBMCs and plays a role in the extrusion of substrates in these cells; therefore, MDR1 has been considered a factor that determines the intracellular concentration of drugs, including immunosuppressants, targeting blood cells. A possible correlation between MDR1 expression in peripheral blood and an ACR rejection episode was determined by immunocytochemical analyses of patients who had undergone a heart and lung transplantation (Kemnitz et al., 1991; Yousem et al., 1993). However, in the case of kidney transplantation, it was reported that MDR1 expression could not predict the occurrence of rejection (Melk et al., 1999). In these studies, cyclosporine was used as the primary immunosuppressant. Grude et al. (Grude et al., 2002) reported that in liver transplant recipients who were administered tacrolimus and cyclosporine, the MDR1 expression level in the total peripheral blood was higher before ACR ($P = 0.054$); further, this value was significantly lower in patients with severe infection ($P = 0.030$) (Grude et al., 2002). These reports did not provide detailed information regarding the blood concentration of immunosuppressants; therefore, it was difficult to establish a direct relationship between these events and the expression level of MDR1 in blood cells. However, these reports suggested a possible relationship between MDR1 expression in blood cells and the susceptibility to calcineurin inhibitors.

We have reported that intestinal MDR1 prevented the intracellular accumulation of orally administered tacrolimus (Goto et al., 2003; Masuda et al., 2003; Masuda et al., 2005; Omae et al., 2005). In addition, the intestinal MDR1

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mRNA level showed a distinct inverse correlation with the concentration/dose ratio of tacrolimus immediately after liver transplantation (Hashida et al., 2001; Masuda et al., 2006). MDR1 is also expressed in the plasma membrane of peripheral leukocytes, mediating the cellular efflux of numerous drugs, including immunosuppressants and anticancer agents (Chaudhary et al., 1992; Klimecki et al., 1994; Ford et al., 2003). Oselin et al. (Oselin et al., 2003) quantified the MDR1 mRNA levels in several types of peripheral blood cells, but the expression levels were not affected by single nucleotide polymorphisms (G2677T and C3435T) of the *MDR1* gene. Based on this information, we hypothesized that a high-expression level of MDR1 in the peripheral blood cells lowers the intracellular concentration of tacrolimus even when the concentration of the drug in the whole blood is sufficient, thereby decreasing its immunosuppressive activity in transplant patients (Fig. 5).

In the present study, we retrospectively examined the effect of MDR1 in the peripheral blood cells on the individualized target concentration of tacrolimus by analyzing the event of ACR as an endpoint in the pediatric LDLT patients. There was a markedly wide interindividual variation in the MDR1 expression level in the peripheral blood cells (Fig. 1A); however, the intraindividual variation was low and not statistically significant (Fig. 1B). Although a low concentration of tacrolimus was considered related to the occurrence of ACR, some patients who were administered a relatively high level of tacrolimus also experienced ACR (Fig. 2). Using *in vitro* sampled whole blood, tacrolimus was found to be mainly distributed in erythrocytes (95–98%) in dog, monkey, and human (Nagase et al., 1994). In addition, the percentage of [^{3}H]dihydro-tacrolimus associated with the lymphocytes of stable

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liver transplant patients (0.8% of whole blood) was significantly higher than that of patients experiencing rejection (0.3%, P = 0.012) (Zahir et al., 2004). Based on these findings, the leukocytic concentration rather than the whole blood concentration of tacrolimus is suggested to be a potent factor affecting the immunosuppressive activity of the drug, and leukocytic MDR1 can be a candidate molecule to decrease the cellular accumulation of tacrolimus (Fig. 5). Comparison of the MDR1 mRNA expression level with the trough concentration of tacrolimus suggested that the target concentration of tacrolimus was higher in patients with a high level of MDR1 mRNA expression in the peripheral blood cells (Fig. 3). Using the *mdr1a/1b* knockout mice, it was revealed that the blood MDR1 acted as a barrier for the cellular accumulation of tacrolimus (Fig. 4). Because of the radioactivity of [^{14}C]tacrolimus, there was a limited decrease in the concentration of tacrolimus and saturation probably occurred. Therefore, the decrease in the concentration of tacrolimus, which is comparable with the clinical situation, may be more affected by the MDR1 expression in the blood cells. These findings suggest that the MDR1 expression level in the peripheral blood cells is a potential pharmacological marker of tacrolimus concentration and can be used to establish the individualized target concentration of this drug.

Therapeutic drug monitoring contributes to the development of individualized pharmacotherapy in patients administered toxic agents, including calcineurin inhibitors. The general therapeutic window of tacrolimus ranges between 5 and 20 ng/mL in transplant patients (Venkataramanan et al., 1995; Masuda and Inui, 2006; Oellerich and Armstrong, 2006). However, the target range always varies with the duration of the postoperative period and the patient status. In patients

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undergoing liver transplantation, the target tacrolimus concentration is between 10 and 20 ng/mL during the 15-day postoperative period. However, the actual level of tacrolimus is relatively low because of its severe adverse effects. We previously reported that at least 7 ng/mL of the average trough concentration of tacrolimus is required immediately after transplantation to prevent ACR (Masuda et al., 2006). However, the ACR episode occurred in 22% of patients in whom the blood tacrolimus concentration was maintained at more than 7 ng/mL. Because tacrolimus targets the leukocytes, the information regarding the concentration of tacrolimus in the whole blood is thought to be insufficient to control the ACR episode in some patients. Therefore, an additional biological marker reflecting the leukocytic concentration of tacrolimus should be identified to establish an individualized target concentration of the drug. In the present study, we have found that a higher target concentration of tacrolimus is required in patients with a high expression level of MDR1 mRNA (>0.05 amol/μg total RNA) than in those with a low expression level (Table 2 and Fig. 3). If the expression level of MDR1 mRNA in the peripheral blood is examined immediately after surgery, we can focus on patients with a high risk of ACR and maintain them on a high level (at least 9 ng/mL) of tacrolimus during the 15-day postoperative period.

In conclusion, the MDR1 mRNA expression level in the peripheral blood is a possible predictor of the susceptibility to tacrolimus; a high expression level of MDR1 might cause ACR, necessitating a higher blood concentration of tacrolimus (Fig. 5). Although further analyses with or without intervention should be investigated in order to establish the clinical significance of blood MDR1 as a pharmacological marker,

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molecular information about MDR1 expression in peripheral blood cells may be useful in the establishment of an individualized target concentration of tacrolimus in children after liver transplantation.

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Footnotes

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Legends for Figures

Fig. 1. Expression levels of MDR1 mRNA in the peripheral blood cells in pediatric patients after living-donor liver transplantation.

A, Histogram of the MDR1 mRNA expression level in the event-free patients (*empty column*) and in those who experienced acute cellular rejection (*filled column*) (n = 44). **B**, Comparison of the MDR1 mRNA levels between postoperative days 3 and 7 (n = 32). The data are expressed as the fold change at postoperative day 7 compared to postoperative day 3. Seven patients were excluded because of high-dose steroid therapy was administered between postoperative days 3 and 7. The blood samples at postoperative day 7 could not be obtained from 5 patients due to patient refusal. **C**, There was no statistical difference with regard to the MDR1 mRNA level in the peripheral blood cells between the event-free patients and those who experienced acute cellular rejection. Statistical analysis was performed using the Mann–Whitney U test (**B** and **C**). However, the P values were greater than 0.05.

Fig. 2. The tacrolimus trough concentrations in the living-donor liver transplant patients who experienced acute cellular rejection (ACR) (E to H) and the event-free patients (A to D) during the15-day postoperative period.

The patients were categorized into the following 4 groups by their MDR1 mRNA level in blood cells at postoperative day 3: below 0.02 amol/μg total RNA (**A** and **E**), between 0.02 and 0.05 amol/μg total RNA (**B** and **F**), between 0.05 and 0.10 amol/μg total RNA (**C** and **G**), and higher than 0.10 (**D** and **H**) amol/μg total RNA. The circles in the panels **E** to **H** represent the tacrolimus trough concentrations on the

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day before the initiation of ACR.

Fig. 3. Average trough concentration of tacrolimus as a function of the mRNA expression level of MDR1 in the blood cells.

The average trough concentration of tacrolimus during the 15-day postoperative period is compared with the logarithmically transformed data representing the mRNA MDR1 levels in the blood cells at postoperative day 3 in the event-free patients (**A**) and in those who experienced acute cellular rejection (**B**). The average concentration of tacrolimus in the 15-day postoperative period is compared between the event-free patients and patients who experienced acute cellular rejection (**C**). Statistical analysis was performed using the Mann–Whitney U test.

Fig. 4. Accumulation of [^{14}C]tacrolimus in PBMCs of wild-type or *mdr1a/1b* knockout mice.

After drawing whole blood from the aorta of FVB-control wild-type mice or *mdr1a/1b* knockout mice, [^{14}C]tacrolimus was spiked in the blood to a final concentration of 10.4 μg/mL (6.4 kBq/mL) and incubated at 37°C for 30 min with gentle shaking. At the end of the incubation, the PBMCs were isolated, dissolved, and subjected to liquid scintillation counting, as described in the methods section. Each column represents the mean ± SD of 7–8 mice. Statistical analysis was performed using the unpaired *t* test. P values less than 0.05 are shown.

Fig. 5. Association between the MDR1 expression level and the tacrolimus

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concentration in leukocytes and whole blood.

The scheme of the hypothesis of the present study is summarized. Most of the tacrolimus in the whole blood is distributed in the erythrocytes (Masuda and Inui, 2006), and the remaining in the plasma and leukocytes. The leukocytic tacrolimus concentration is sufficient for immunosuppression (*large arrow*) when MDR1 expression levels are low (**A**). However, with the same concentration of tacrolimus in the whole blood, the leukocytic level of tacrolimus is decreased to a level that is insufficient for immunosuppression (*small arrow*) when the MDR1 expression levels are high (**B**). Therefore, in the case of high expression levels of MDR1, a higher concentration of tacrolimus in the whole blood is required to increase its leukocytic level and enable subsequent sufficient immunosuppression (**C**).

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Table 1. Demographics of patients

	Control (event-free)		Acute cellular rejection	
Number of Patients		23		21
Age (years)		0.25–12		0.5–14.1
Primary Disease	Biliary atresia	16	Biliary atresia	15
	with liver tumor	1	Alagille syndrome	2
	with Alagille syndrome	1	Re-LDLT*	1
	Re-LDLT	3	Primary sclerosing cholangitis	1
	Hyperpropionemia	2	Byler disease	1
			Wilson disease	1
Blood Type	Identical	18	Identical	16
	Compatible	5	Compatible	5

*LDLT, living-donor liver transplantation

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Table 2. Effect of tacrolimus trough level and MDR1 expression on the occurrence of acute cellular rejection

	Control (event-free)	Acute cellular rejection	χ^2 test	Odds ratio
	Number	Number	P value	(95% confidence interval)
All patients				
>9 ng/mL*	14	2	0.0004	14.77
<9 ng/mL	9	19		(2.752–79.33)
Patients with blood MDR1 mRNA levels below 0.05 amol/μg total RNA†				
>9 ng/mL	4	0	0.0154	not available
<9 ng/mL	6	12		
Patients with blood MDR1 mRNA levels higher than 0.05 amol/μg total RNA				
>9 ng/mL	10	2	0.0112	11.66
<9 ng/mL	3	7		(1.527–89.12)

*, The patients were categorized by the average trough concentration of tacrolimus during the 15-day postoperative period. The trough concentrations of tacrolimus during high-dose steroid pulse therapy were excluded.

†, The patients were categorized by the median value of the MDR1 mRNA expression level in blood cells on postoperative day 3.

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Fig. 1.

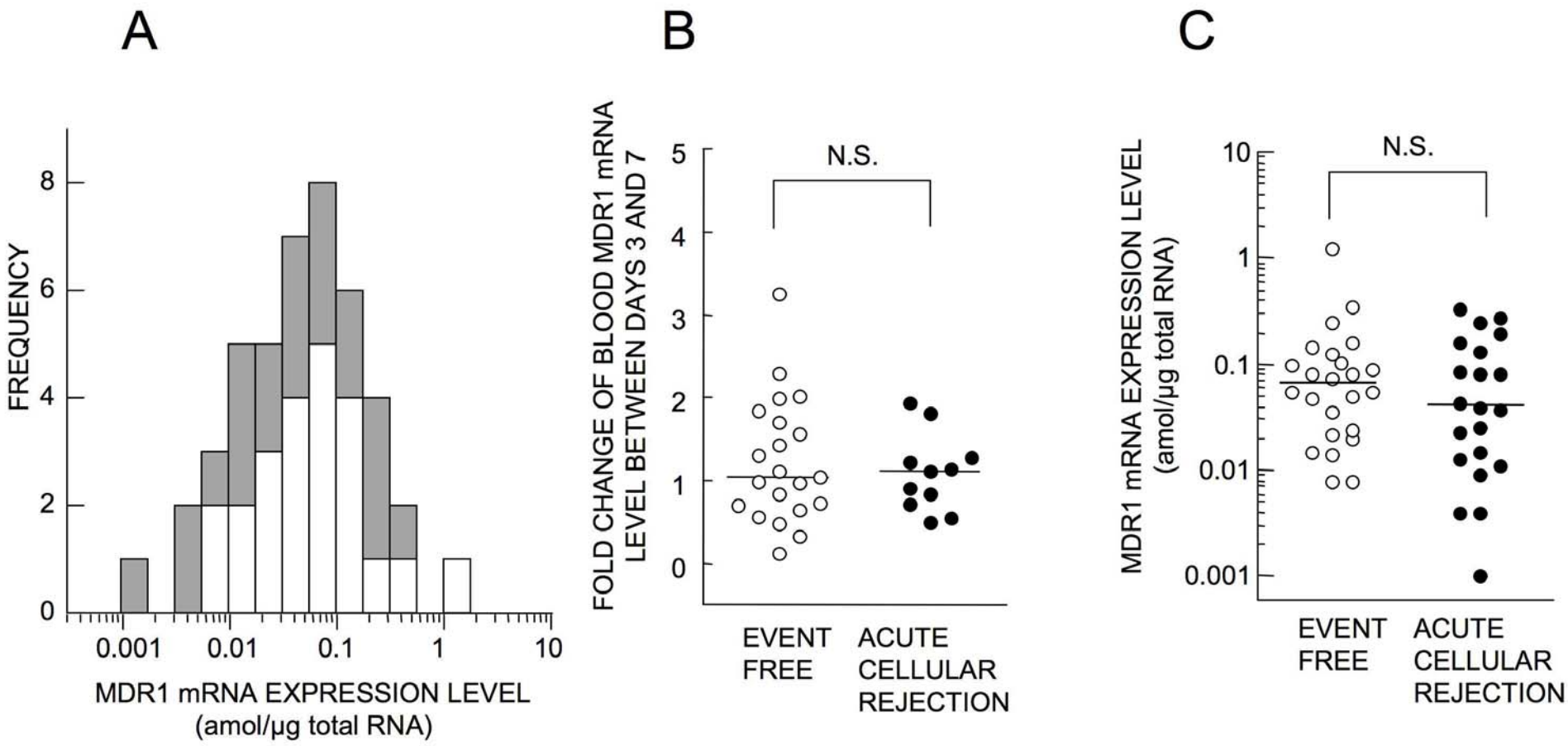

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Fig. 2.

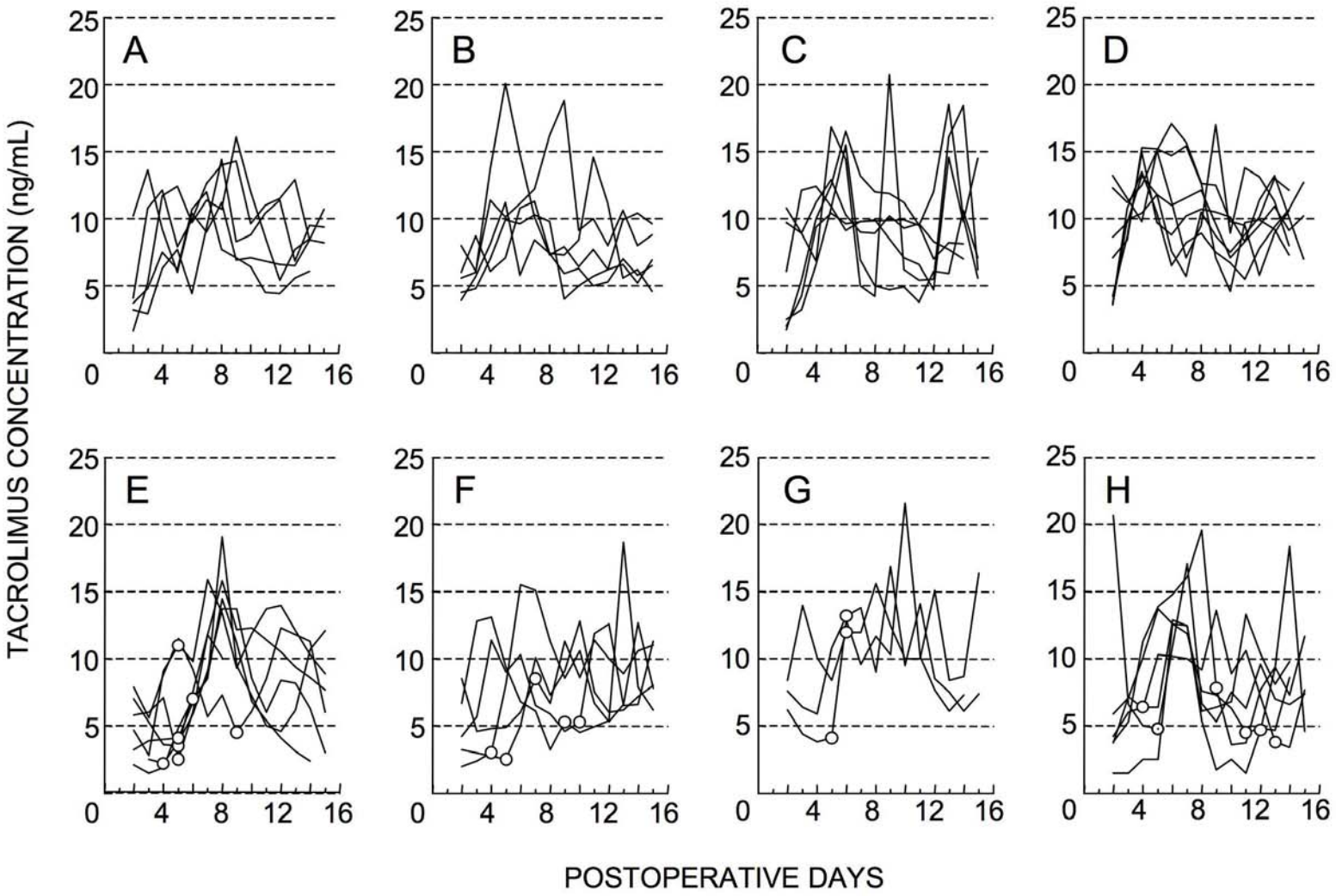

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Fig. 3.

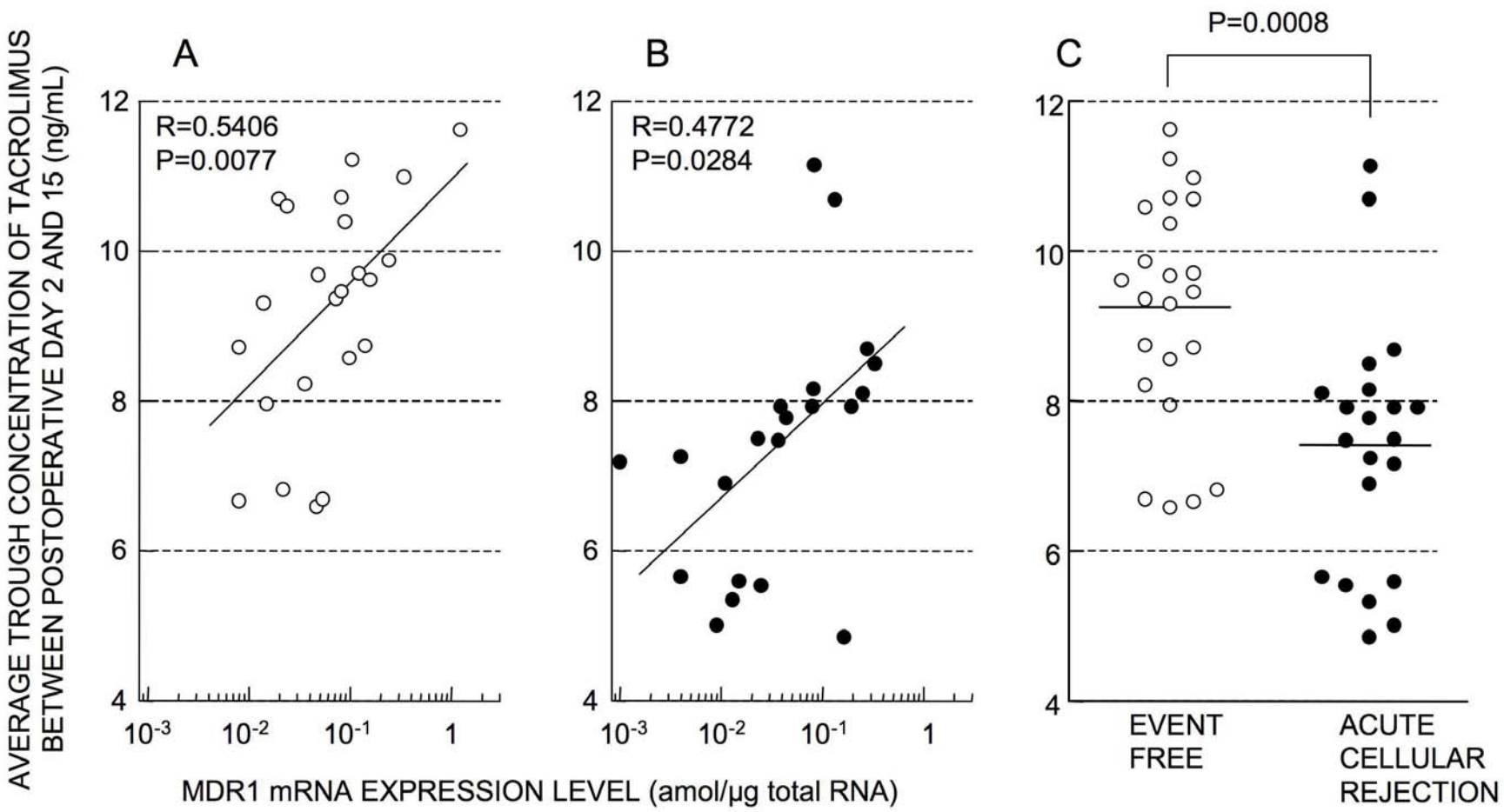

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Fig. 4.

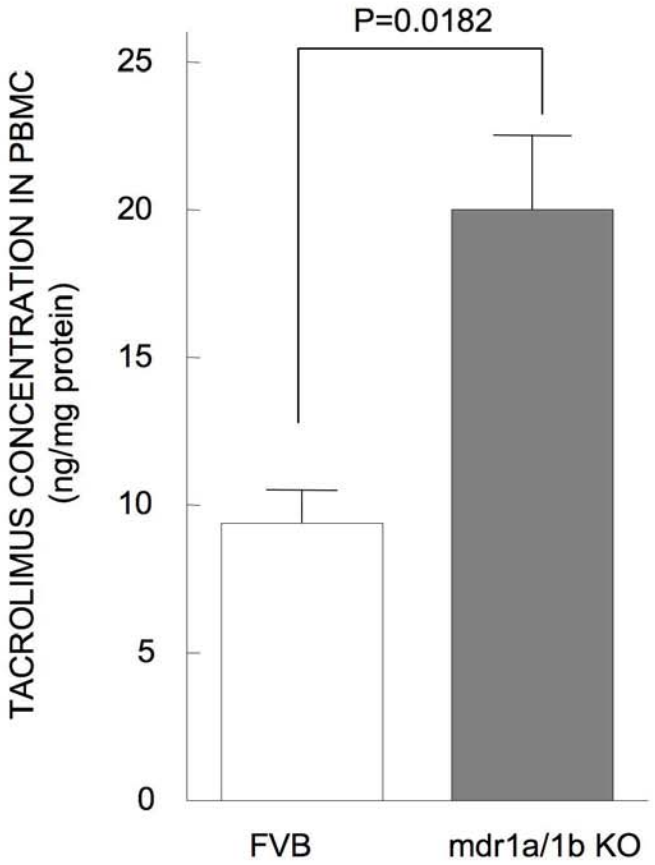

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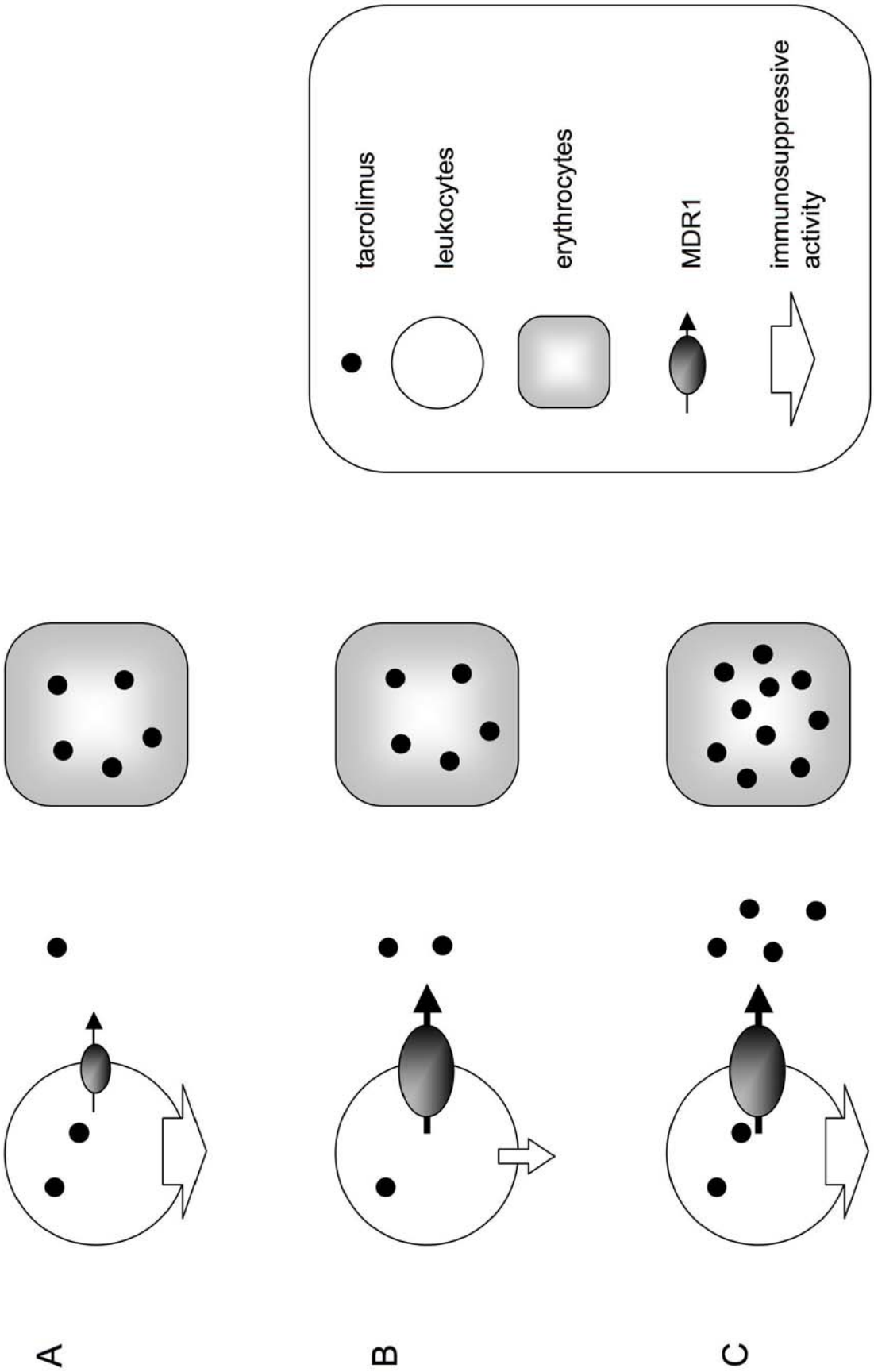
Fig. 5.



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