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# Mycophenolic Acid Inhibits IL-2-Dependent T Cell Proliferation, But Not IL-2-Dependent Survival and Sensitization to Apoptosis<sup>1</sup>

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Mycophenolic acid (MPA), the active metabolite of the immunosuppressive drug mycophenolate mofetil, is a selective inhibitor of inosine 5'-monophosphate dehydrogenase type II, a de novo purine nucleotide synthesis enzyme expressed in T and B lymphocytes and up-regulated upon cell activation. In this study, we report that the blockade of guanosine nucleotide synthesis by MPA inhibits mitogen-induced proliferation of PBL, an effect fully reversed by addition of guanosine and shared with mizoribine, another inhibitor of inosine 5'-monophosphate dehydrogenase. Because MPA does not inhibit early TCR-mediated activation events, such as CD25 expression and IL-2 synthesis, we investigated how it interferes with cytokine-dependent proliferation and survival. In activated lymphoblasts that are dependent on IL-2 or IL-15 for their proliferation, MPA does not impair signaling events such as of the extracellular signal-regulated kinase 2 and Stat5 phosphorylation, but inhibits down-regulation of the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup>. Therefore, in activated lymphoblasts, MPA specifically interferes with cytokine-dependent signals that control cell cycle and blocks activated T cells in the mid-G<sub>1</sub> phase of the cell cycle. Although it blocks IL-2-mediated proliferation, MPA does not inhibit cell survival and Bcl-x<sub>L</sub> up-regulation by IL-2 or other cytokines whose receptors share the common  $\gamma$ -chain (CD132). Finally, MPA does not interfere with IL-2-dependent acquisition of susceptibility to CD95-mediated apoptosis and degradation of cellular FLIP. Therefore, MPA has unique functional properties not shared by other immunosuppressive drugs interfering with IL-2R signaling events such as rapamycin and CD25 mAbs. *The Journal of Immunology*, 2002, 169: 2747–2755.

**M**ycophenolate mofetil (MMF)<sup>3</sup> is an immunosuppressant presently used for the prevention or treatment of acute rejection in organ transplantation (1). Its immunosuppressive and anti-inflammatory properties are currently under evaluation in several autoimmune disorders. MMF is a semi-synthetic derivative of mycophenolic acid (MPA), a selective noncompetitive inhibitor of inosine 5'-monophosphate dehydrogenase (IMPDH). IMPDH is a rate-limiting enzyme in the de novo synthesis of guanine ribo- and 2'-deoxyribonucleotides. Mitogenic stimulation of T lymphocytes results in a marked increase in IMPDH activity and a 5-fold increase in the guanine nucleotide pool (2, 3). MPA is a more potent inhibitor of type II IMPDH, which is expressed in activated lymphocytes, than of the type I expressed in most cell types (4, 5).

Blockade of IMPDH by MPA was shown to deplete the guanosine (Gua) pool in lymphocytes (6) and to inhibit T and B cell proliferation, differentiation of alloreactive cytotoxic T cells, and Ab responses (7–9). More recently, MMF was shown to impair maturation of murine dendritic cells, suggesting that MMF can also affect APC functions (10). Inhibition of T lymphocyte proliferation was shown to result from a blockade of activated lymphocytes in early- to mid-G<sub>1</sub> phase of the cell cycle, resulting from a lack of induction of cyclin D/cyclin-dependent kinase (CDK) 6 and impaired degradation of the CDK inhibitor p27<sup>Kip1</sup> (9). Down-regulation of the CDK inhibitor p27<sup>Kip1</sup> was shown to depend on the binding of IL-2 to its high-affinity trimeric receptor (11–13). Earlier studies with the immunosuppressive agent mizoribine (MZB), another inhibitor of IMPDH, have shown that depletion of guanine nucleotides in T lymphocytes inhibits the entry into S phase of the cell cycle, but does not alter early G<sub>1</sub> events such as expression of c-Myc, IL-2, or IL-2R (14). Therefore, we investigated whether IMPDH inhibitors, including MPA, could affect T cell proliferation by interfering with IL-2-dependent signaling events that are required for p27<sup>Kip1</sup> degradation and progression to the S phase of the cell cycle. We next studied whether other functions of IL-2 toward activated T cells are affected by MPA. Indeed, promotion of T cell proliferation is the key activity attributed to IL-2. Most of the currently used immunosuppressive agents are targeted at the suppression of T cell clonal expansion, by transcriptional inhibition of IL-2 gene expression (e.g., calcineurin inhibitors), by interference with IL-2 binding to its receptor (e.g., CD25 mAbs), or with intracellular signals (e.g., rapamycin (RPM)). However, the biological responses elicited by IL-2 appear to be much broader than originally thought. IL-2, as well as other cytokines such as IL-4, IL-7, and IL-15 whose receptors share the common  $\gamma$ -chain, provides a survival signal for activated T cells

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<sup>3</sup> Abbreviations used in this paper: MMF, mycophenolate mofetil; CDK, cyclin-dependent kinase; Gua, guanosine; IMPDH, inosine 5'-monophosphate dehydrogenase; MPA, mycophenolic acid; MZB, mizoribine; RPM, rapamycin; ERK, extracellular signal-regulated kinase; cFLIP, cellular FLIP.

(15) through the induction of the anti-apoptotic genes Bcl-2 and Bcl-x<sub>L</sub> (16). More surprising was the phenotype of IL-2-deficient mice, showing that IL-2 is not essential for the generation, clonal expansion, and differentiation of lymphocytes to effector cells, but rather has a unique role in preventing the accumulation of activated T cells (17, 18). Indeed, these mice develop massive enlargement of peripheral lymphoid organs associated with polyclonal T cell expansion, which is correlated with impaired activation-induced cell death (19) and the failure to generate functional CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells (20, 21).

The use of currently available immunosuppressive drugs has markedly decreased the incidence of acute rejection in organ transplantation, but none of these agents has been shown to induce tolerance. Inhibition of T cell clonal expansion is not sufficient to reach this goal. Apoptosis of alloreactive T cells seems to favor the development of immune tolerance to allografts (22). Therefore, drugs interfering with IL-2 synthesis or signaling should be evaluated not only for their antiproliferative activities, but also for other IL-2-dependent activities, including activation-induced apoptosis (23, 24).

In order to analyse how MPA interferes with cytokine-mediated signals, we used lymphoblasts derived from PHA-stimulated PBL cultured with IL-2. Those cells can be induced to divide by addition of IL-2 or IL-15 and their survival requires the presence of either IL-2, IL-4, IL-7, or IL-15. This model is suited for studying drug interference with proliferation vs cell survival. Furthermore, we investigated whether MPA interferes with the priming of activated T cells to CD95-mediated apoptosis by comparison with RPM, another immunosuppressive drug known to interfere with IL-2R signaling events.

## Materials and Methods

### *Abs and reagents*

MPA was kindly provided by Roche Bioscience (Palo Alto, CA). PHA, Gua, and RPM were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Recombinant human IL-2 was obtained from Chiron (Suresnes, France). Recombinant human IL-4, IL-7, and IL-15 were obtained from PeproTech (TEBU; Le-Perray-en-Yvelines, France). The agonist anti-CD95 mAb (7C11, IgM) was purchased from Immunotech (Marseille, France). The CD3 mAb OKT3 was from Orthoclone (Levallois-Perret France). The CD28 mAb (clone CD28.1) was purchased from DAKO (Trappes, France). The CD25 mAb ARIL-2 (IgG1) was a gift from Dr. Carcagne (Biomérieux, Lyon, France).

### *Cell preparation and culture*

PBL were collected from healthy donors in the presence of sodium citrate. Blood was defibrinated, then mononuclear cells were isolated by centrifugation on a layer of Histopaque (Dutcher, Brumath, France). Those suspensions contained 64.4 ± 2.0% T lymphocytes, 7.5 ± 1.2% B lymphocytes, 16.1 ± 1.9% NK cells, and 0.8 ± 0.4% monocytes as defined by expression of CD3, CD20, CD56, and CD14, respectively.

PBL were resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and stimulated in a humid atmosphere containing 5% CO<sub>2</sub>.

Activated lymphoblasts were obtained by activation of PBL for 3 days with PHA (5 µg/ml) and then 7 days with IL-2 (50 U/ml). These long term-activated lymphoblasts were mainly (62%) CD8<sup>+</sup> cells, with 10% of CD4<sup>+</sup> and 20% of NK cells.

### *CFSE staining*

To follow cell division, cells (1 × 10<sup>7</sup>/ml) were pulsed with 1 µM fluorescent dye CFSE (Molecular Probes, Montluçon, France) in 2% FCS medium at 37°C. Then cells were washed and resuspended in medium at 1 × 10<sup>6</sup>/ml. After 3 or 6 days, cells were resuspended in PBS containing 2% BSA and 0.2% NaN<sub>3</sub> (PBS/BSA/azide) and fixed with 1% formaldehyde in PBS/BSA/azide buffer. CFSE staining (FL1-height) was analyzed by FACS (I. Ex. Max. 488 nm; I. Em. Max. 525 nm) with CellQuest software (BD Biosciences Pont de Claix, France).

### *[<sup>3</sup>H]TdR incorporation*

During the last 8 h of culture, cells were pulsed with [<sup>3</sup>H]TdR (Amersham, Saclay, France) at 0.5 µCi/well. [<sup>3</sup>H]TdR uptake was measured using a Packard direct beta counter (Packard Instrument, Meriden, CT) after harvesting.

### *Immunofluorescence staining*

After a wash with PBS/BSA/azide, cells (5 × 10<sup>5</sup>) were incubated with 5 µl of FITC-conjugated mAb for 30 min at 4°C. After washes, cells were resuspended in PBS/BSA/azide buffer and analyzed by FACS.

FITC-conjugated anti-CD69, -CD25, -CD154, -CD71 mAbs and PE-conjugated anti-CD4 and -CD8 mAbs were obtained from BD Biosciences.

### *Assay of IL-2 in culture supernatant*

Cell-free supernatants were harvested and IL-2 concentration was determined by ELISA. Briefly, serial dilutions of culture supernatant (100 µl) were added to duplicate wells coated with anti-IL-2 mAb (Duoset; R&D Systems, Oxon, U.K.). After incubation for 1 h at 37°C, 100 µl of biotinylated polyclonal rabbit anti-human IL-2 (Duoset; R&D Systems) was added to the wells. After 1 h at 37°C, plates were incubated for 15 min at 37°C with 100 µl of peroxidase-conjugated streptavidin. After washes, orthophenyldiamine (100 µl; Sigma-Aldrich) was added to each well and, after 15 min of incubation, the reaction was stopped by addition of 2N H<sub>2</sub>SO<sub>4</sub>. Absorbance at 620 nm was recorded on a Multiskan MCC/340 (Labsystems, Lugano, Switzerland).

### *Analysis of cyclin E and p27<sup>Kip1</sup> expression*

After starving of activated cells for 16 h, cells were stimulated for the indicated time with IL-2 50 ng/ml. MPA (10 µM) or 500 nM RPM were added at the onset of the starving. Cells were washed with ice-cold PBS and collected in microcentrifuge tubes for lysis. The lysis buffer contained 50 mM HEPES (pH 7.2), 150 mM NaCl, 100 mM EDTA, 100 mM EGTA, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 300 µg/ml benzamide, 75 µg/ml PMSF, and 10 µg/ml tosylphenylalaninechloromethylketone. After centrifugation at 50,000 rpm for 1 h, the protein content in the supernatant was assayed by the Bradford method using Coomassie dye (Bio-Rad, Marnes-la-Coquette, France). Equal amounts of protein were precipitated in acetone at 4°C overnight and separated on a 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Ecquevilly, France). The membrane was blocked with 5% nonfat milk in TBST and incubated 1 h with primary Ab in blocking solution. Membranes were then washed five times with TBST and incubated 45 min with the appropriate secondary Ab. Detections were performed using the ECL chemiluminescence system (Amersham). Anti-cyclin E and anti-p27<sup>Kip1</sup> (F-8) mAbs were obtained from Santa Cruz Biotechnology (TEBU; Santa Cruz, CA). Biotinylated secondary Ab was purchased from BD PharMingen (Pont de Claix, France). HRP-conjugated streptavidin was obtained from Amersham. Equal amount of proteins loaded have been controlled by probing membrane with β-actin mAb (Sigma-Aldrich).

### *Analysis of Stat5 and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation*

After 6 h of starving, activated cells were stimulated for the indicated time with IL-2 50 ng/ml. MPA (10 µM) or 500 nM RPM were added at the onset of the starving. After treatment, cells were washed in ice-cold PBS. For Stat5 activation, cells (5 × 10<sup>6</sup> per sample) were lysed in SDS sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 2% 2-ME). Proteins were resolved in 7.5% SDS-PAGE and electroblotted. Blots were probed using an anti-phospho-Stat5 (Tyr<sup>694</sup>) mAb (Cell Signaling Technology, Ozyme, St. Quentin en Yvelines, France), and anti-Stat5 mAb (Santa Cruz Biotechnology). For ERK1/2 phosphorylation, cells (40 × 10<sup>6</sup> per sample) were lysed in lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 10 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM PMSF, 2.5 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF) and protein supernatant was separated on 12% SDS-PAGE and electroblotted. Blot was probed using anti-phospho-ERK1/2 mAb (Upstate Biotechnology, Euromedex, Mundolsheim, France) or anti-ERK1/2 Ab (Upstate Biotechnology).

### *Analysis of cFLIP expression*

Activated PBL (10<sup>7</sup> per sample) were washed twice with ice-cold PBS and resuspended in lysis buffer (10 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton-X 100, 10 mM EDTA, supplemented with 10 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM PMSF) 15 min at 4°C. Thirty micrograms of protein were separated on 12% SDS-PAGE and transferred to nitrocellulose

membrane. Blot was probed using an anticellular (cFLIP) FLIP mAb kindly provided by Prof. P. H. Krammer (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Equal amounts of proteins loaded have been controlled by probing membrane with  $\beta$ -actin mAb.

#### Measurement of apoptosis

Exposure of phosphatidylserine was quantified by surface annexin V staining. Cells were resuspended in binding buffer, incubated with FITC-conjugated annexin V (Bender MedSystems, Vienna, Austria) for 5 min then stained with propidium iodide (1  $\mu$ g/ml). Apoptosis was defined as annexin V<sup>+</sup> cells.

#### Intracellular Bcl-x<sub>L</sub> staining

Bcl-x<sub>L</sub> protein expression was investigated after permeabilization of the cell membrane with Cytofix/Cytoperm kit (BD Pharmingen). Briefly, cells (1  $\times$  10<sup>6</sup>) were resuspended in Cytofix/Cytoperm solution. After 20 min at 4°C and one wash with Perm/wash buffer, cells were resuspended in anti-Bcl-x<sub>L</sub> mAb (Southern Biotechnology Associates, Monroville, France) or control isotype diluted in the Perm/wash buffer and incubated for 30 min at 4°C. After washes, cells were resuspended in PBS 2% paraformaldehyde and analyzed by FACS.

## Results

### MPA inhibits T cell proliferation

To assess how MPA could interfere with the expansion of activated lymphocytes, human PBL were activated with the mitogenic lectin PHA (5  $\mu$ g/ml) in the presence or absence of MPA (10  $\mu$ M), and the fluorescent dye CFSE was used to track cell division by FACS. After 3 days in the presence of PHA, 42% of lymphocytes had undergone one to three divisions, and only 21% of the cells remained undivided after 6 days. Addition of MPA at the onset of the culture inhibited cell division (Fig. 1A). Inhibition was dose-dependent, starting at 0.1  $\mu$ M and being maximal at 10  $\mu$ M (data not shown). Gua at 100  $\mu$ M, a concentration previously shown to replete guanine nucleotide pool via the salvage pathway (1), prevented the inhibitory effect of MPA and allowed cell division to proceed. Of note, in the presence of MPA and Gua, cell division was delayed suggesting a potential effect of MPA not reversed by addition of Gua. The proliferation induced by other activators, including anti-CD3 and anti-CD28 mAbs, was also completely inhibited by MPA (Fig. 1C). The effect of MPA affected proliferation of both CD4 and CD8 subpopulations (Fig. 1B). In parallel, cell cycle progression of PHA-activated lymphocytes was studied by measurement of DNA content at a single cell level. Treatment with MPA completely abolished the progression into the S phase and cells were arrested at the G<sub>0</sub>/G<sub>1</sub> phase and addition of Gua reversed the G<sub>1</sub> blockade (data not shown).

### MPA inhibits blastogenesis but does not interfere with initial events of T cell activation

Progression from quiescent state (G<sub>0</sub>) into and through the G<sub>1</sub> phase of the cell cycle is characterized by RNA synthesis, decondensation of the chromatin, cellular enlargement, and synthesis of new proteins. Cellular enlargement as well as up-regulation of the activation marker CD69, the IL-2R  $\alpha$ -chain CD25, CD154, and the transferrin receptor CD71, were analyzed by FACS (Fig. 2). As expected, PHA stimulation induced an increase in the forward scatter of the cells and triggered CD69, CD25, CD154, and CD71 expression. CD69 and CD25 were strongly up-regulated at day 1, whereas CD154 and CD71 were only slightly induced at day 1 and then highly expressed on most cells at day 3 (Fig. 2). Addition of MPA did not modify up-regulation of the early G<sub>1</sub> markers CD69 and CD25 at days 1 or 3, and the slight expression of CD154 and CD71 at day 1. In contrast at day 3, up-regulation of CD71 and CD154 expression was strongly inhibited by MPA. At day 3, MPA also completely inhibited the development of lymphoblasts characterized by an increase in size and cellular granularity.

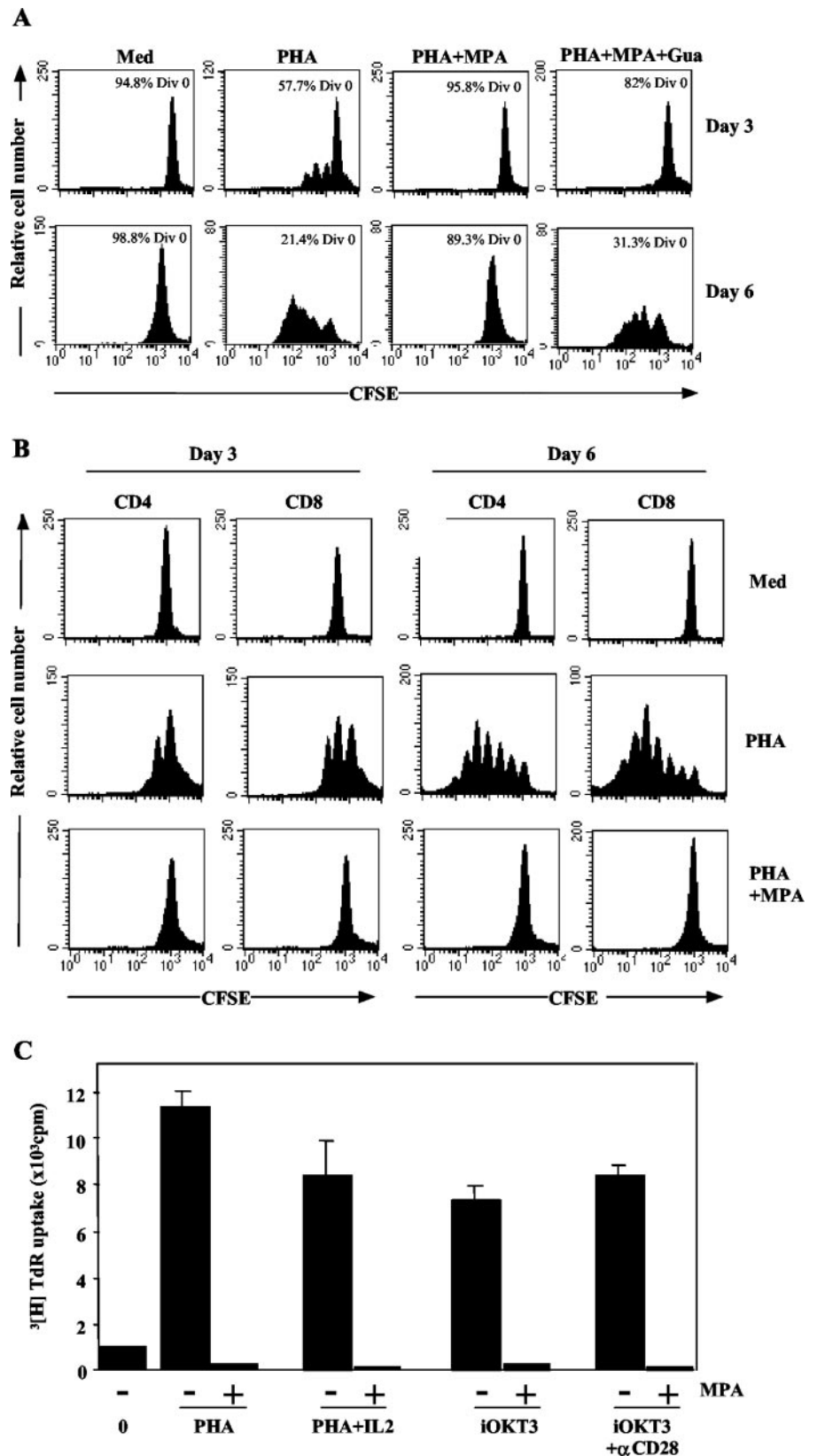
One of the key events during G<sub>1</sub> phase is the synthesis of IL-2 by activated T lymphocytes. IL-2 production is required for progression of T cells through G<sub>1</sub> into the S phase of the cell cycle (25). Because MPA inhibits cell proliferation it was important to test whether IL-2 production was affected. Under PHA stimulation, [<sup>3</sup>H]TdR uptake rose progressively until day 4, and the IL-2 level in the supernatant was maximal at day 2 and declined thereafter (Fig. 3). As expected, [<sup>3</sup>H]TdR uptake was completely inhibited in the presence of MPA (Fig. 3A), but the IL-2 level in supernatants was not decreased and remained high between days 2 and 4 (Fig. 3B). These data suggest that IL-2 synthesis and secretion during the G<sub>1</sub> phase were not decreased by MPA and that despite normal expression of CD25, IL-2 was not consumed because MPA had prevented the generation of daughter T cells expressing high levels of IL-2R.

### MPA does not induce T cell apoptosis following T cell activation

We next analyzed whether MPA affects cell survival following mitogenic activation. In those experiments, we also tested the effect of RPM and anti-CD25 Ab (ARIL-2), two other immunosuppressive agents, which inhibited proliferation (Fig. 4A) without interfering with initial events of T cell activation such as IL-2 production (Ref. 13 and data not shown). Counts of viable cells in the presence of PHA showed, after an initial decrease from 1  $\times$  10<sup>6</sup> cells/ml at day 0 to 0.68  $\times$  10<sup>6</sup> cells/ml at day 2, a progressive increase to reach 2.1  $\times$  10<sup>6</sup> cells/ml at day 5. Cell numbers in the presence of medium alone remained stable,  $\sim$ 1  $\times$  10<sup>6</sup> cells/ml. In the presence of MPA, cell numbers remained stable at 0.65  $\times$  10<sup>6</sup> cells/ml from days 1 to 3 and slightly decreased to 0.55  $\times$  10<sup>6</sup> cells/ml at days 4 and 5 (Fig. 4B). Similarly to MPA, RPM and anti-CD25 did not prevent the initial decrease following mitogenic activation. However, after day 2, cell counts increased up to 0.95  $\times$  10<sup>6</sup> cells/ml in the presence of RPM and 1.1  $\times$  10<sup>6</sup> cells/ml in the presence of anti-CD25 Abs. Such an increase might reflect an incomplete blockade of cell division by RPM and anti-CD25 Abs as suggested by the low percentage of dividing cells observed in Fig. 4A. In parallel, we followed the percentage of apoptotic cells by annexin V labeling. As shown in Fig. 4C, percentage of apoptotic cells remained stable between 25 and 35% during the five-day culture period in the presence of PHA alone or PHA + RPM and PHA + anti-CD25 Abs. The percentage of apoptotic cells was slightly over 40% at days 4 and 5 in the presence of MPA (Fig. 4C). Therefore, MPA inhibited T cell proliferation without a major decrease of activated T cell viability at days 4 and 5.

### MPA inhibits IL-2- and IL-15-dependent T cell proliferation

Having demonstrated that the antiproliferative effect of MPA does not result from a defective IL-2 synthesis, we next investigated the effect of MPA as well as MZB, another IMPDH inhibitor, on T cell proliferation stimulated exclusively by IL-2 without TCR triggering. For such experiments, IL-2-dependent T lymphoblasts were obtained by culture of PBL for 3 days in the presence of PHA followed by 7 days with IL-2. These long term-activated lymphoblasts were mainly CD8<sup>+</sup> cells. They expressed high levels of  $\gamma$ -chain (CD132), whereas expression of  $\alpha$ -(CD25) and  $\beta$ -(CD122) chains of IL-2R was heterogeneous (data not shown). Those cells were mostly in the G<sub>1</sub> phase of the cell cycle (data not shown), and did not incorporate [<sup>3</sup>H]TdR. IL-2 or IL-15, but not IL-4 or IL-7, strongly stimulated their proliferation (Table I). MPA or MZB, added concomitantly with IL-2 or IL-15, inhibited cytokine-induced proliferation in those cells (Table I). The inhibitory effect of MPA and MZB was compared to that of RPM, an immunosuppressive agent previously shown to interfere with IL-2-driven signaling pathway. As shown in Table I, a

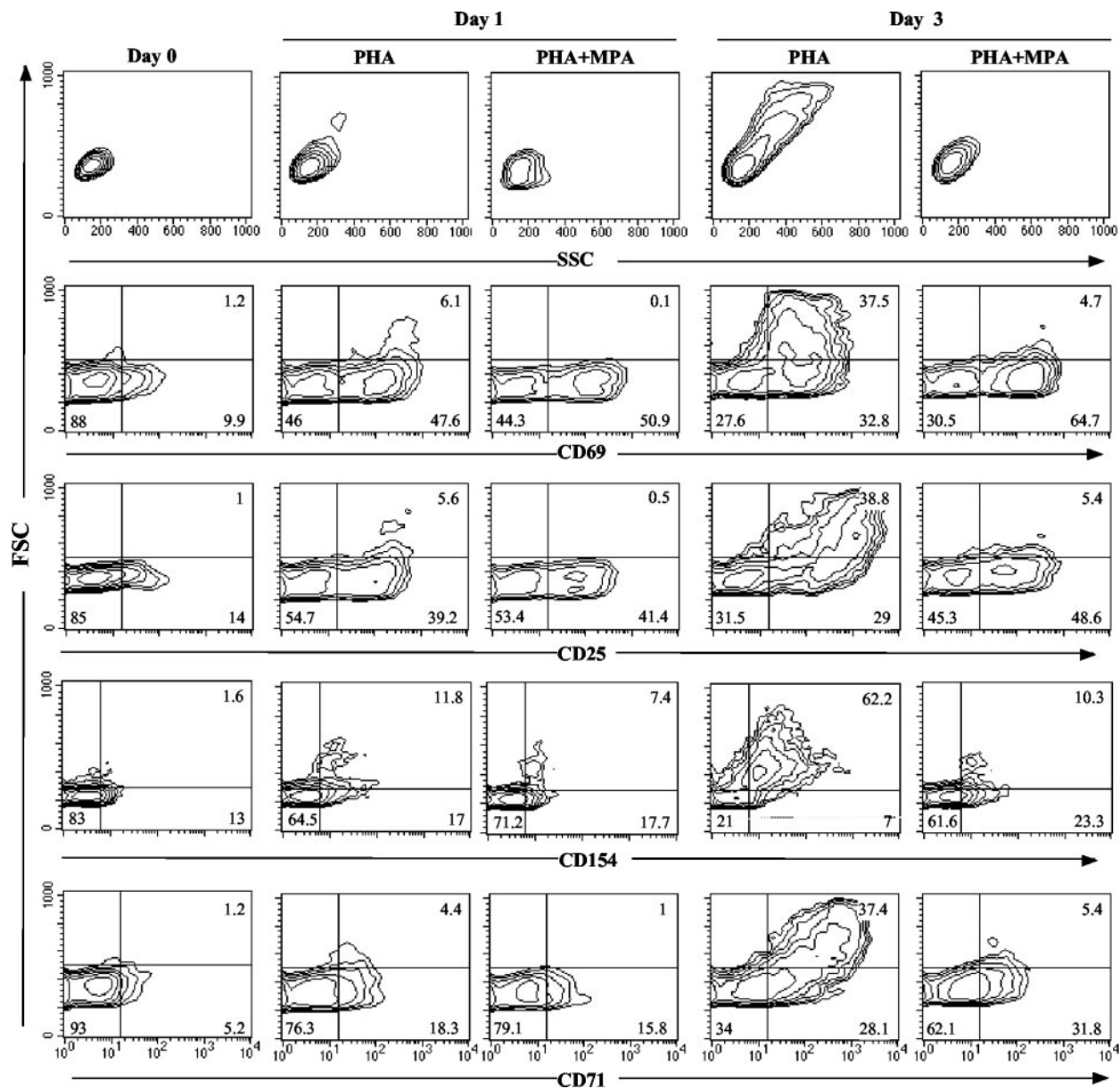


**FIGURE 1.** Inhibition of T cell proliferation by MPA. PBL were labeled by CFSE as described in *Materials and Methods*, and activated by PHA (5  $\mu$ g/ml) in the presence or absence of MPA (10  $\mu$ M) with or without Gua (100  $\mu$ M). At the indicated time of culture, cells were harvested and cell divisions were analyzed by FACS. *A*, Percentage of cells, that did not divide, is indicated on the figure. Results are representative of three individual experiments. *B*, After staining with PE-conjugated anti-CD4 or -CD8 mAbs, cells were electronically gated on CD4<sup>+</sup> or CD8<sup>+</sup> T cells and the expression of CFSE was examined. Histograms are representative of two independent experiments. *C*, PBL were activated for 3 days by the indicated mitogens in the presence or absence of MPA (10  $\mu$ M). [<sup>3</sup>H]TdR incorporation was measured during the last 8 h of culture. Values are the mean  $\pm$  SEM from triplicate measurement of one experiment from a total of three showing similar results.

similar inhibition of IL-2- or IL-15-dependent proliferation was observed in the presence of RPM.

Down-regulation of the CDK inhibitor p27<sup>Kip1</sup> is a critical event in the control of G<sub>1</sub>- to S-phase transition (26) and was shown to be dependent on IL-2 signaling (27), possibly through the p21<sup>ras</sup>/Raf/MEK/ERK pathway (28). Therefore we analyzed the expres-

sion of the CDK inhibitor p27<sup>Kip1</sup> in IL-2-stimulated lymphoblasts in the presence or absence of MPA. As shown in Fig. 5A, IL-2 induced the down-regulation of p27<sup>Kip1</sup> within 24 h. Degradation of p27<sup>Kip1</sup> did not occur when cells were stimulated with IL-2 in the presence of MPA. As expected a similar inhibition of p27<sup>Kip1</sup> down-regulation was observed in presence of RPM (Fig. 5A) (13,



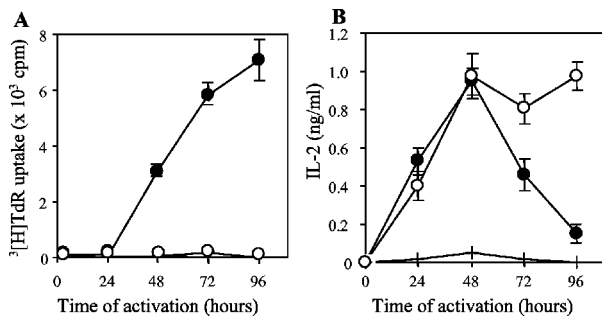
**FIGURE 2.** Effect of MPA on blastogenesis. PBL were stimulated with PHA alone or in the presence of MPA (10  $\mu$ M). At the indicated time, FSC (forward scatter), SSC (side scatter), and expression of activation markers (CD69, CD25, CD154, and CD71) were analyzed by FACS. Results are representative of one among five independent experiments for CD69, CD25, and CD71 and two for CD154.

29). Cyclin E is expressed in the late G<sub>1</sub> phase of the cell cycle and is essential for progression through the S phase. In activated T lymphoblasts, cyclin E expression was high and was not altered by IL-2 stimulation alone or in the presence of MPA and RPM (Fig. 5A). We did not observe any modification of p21<sup>ras</sup> activation (data not shown) and ERK2 phosphorylation (Fig. 5B) following IL-2R triggering when cells were pretreated with either MPA or RPM. These results suggest that the p21<sup>ras</sup>/Raf/MEK-1/ERK2 pathway is functional, up to the activation of ERK2 in MPA- or RPM-treated lymphoblasts.

The involvement of Stat5 protein in the control of IL-2-stimulated proliferation, remains controversial (30–32). In our experiments, Stat5 was not phosphorylated in long term-activated lymphoblasts starved for 6 h in the presence of medium alone (Fig. 5C). Re-stimulation with IL-2 induced a rapid phosphorylation of the protein which was maximal after 30 min (Fig. 5C). Pretreatment of cells with either MPA or RPM did not inhibit IL-2-induced Stat5 phosphorylation (Fig. 5C).

#### MPA and RPM do not affect IL-2-mediated survival signal

Another important function of IL-2 is to promote the survival of activated T lymphocytes, a property previously correlated with the expression of Bcl-2 and related proteins (16, 32, 33). Therefore, we investigated whether MPA would also inhibit IL-2-mediated survival signals in activated T cells. T cells previously activated by PHA and cultured with IL-2 rapidly underwent apoptosis in the absence of added cytokines. As shown in Fig. 6A, 43% of cells were annexin V-positive at 24 h and 59% after 72 h. Addition of IL-2 (Fig. 6A), as well as IL-4, -7, or -15 (data not shown) prevented apoptosis. Interestingly, addition of MPA, at the concentration previously shown to inhibit IL-2-stimulated proliferation, did not interfere with IL-2, IL-4, IL-7, or IL-15-mediated survival (Fig. 6A and data not shown). In agreement with those observations, induction of Bcl-x<sub>L</sub> expression in the presence of IL-2 was not inhibited by MPA (Fig. 6B). RPM, like MPA, did not interfere with IL-2-mediated T cell survival and up-regulation of Bcl-x<sub>L</sub>.



**FIGURE 3.** MPA did not prevent IL-2 level. **A**, Kinetic of [<sup>3</sup>H]TdR uptake in medium alone (+) with PHA (●) or PHA + MPA (○). **B**, PBL were incubated in medium alone (+) with PHA (●) or PHA + MPA (○). At the indicated time, culture supernatants were harvested and the IL-2 level was measured by ELISA. Values are the mean ± SEM of three independent experiments.

#### RPM, but not MPA, prevents acquisition of sensitivity to CD95-mediated apoptosis

A third important function of IL-2 is to sensitize activated T cells to CD95-mediated apoptosis (19, 34). Reconstitution of IL-2Rβ<sup>-/-</sup> T cells with wild-type or mutant versions of the IL-2Rβ-chain demonstrated that this function of IL-2 can be uncoupled from cell growth and survival (32). IL-2 is required for degradation of cFLIP during T cell activation to render cells sensitive to CD95-mediated apoptosis (19). Previous reports have demonstrated that immunosuppressive agents that block IL-2 synthesis such as cyclosporin A or FK506, and also RPM which interferes with IL-2

**Table I.** MPA and RPM blocked IL-2- and IL-15-dependent T cell proliferation

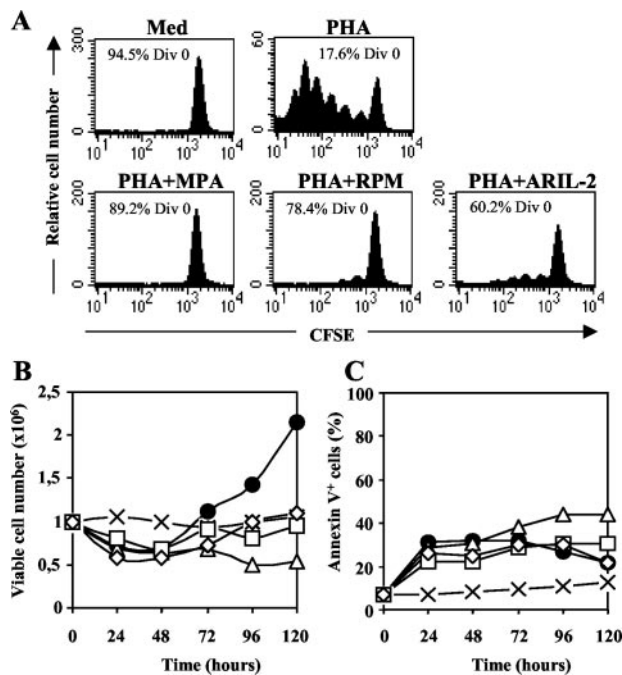
	[ <sup>3</sup> H]TdR Uptake (×10 <sup>3</sup> cpm) <sup>a</sup>		
	24 h	48 h	72 h
Medium	0.21 ± 0.02	0.26 ± 0.01	0.10 ± 0.01
IL-2	0.80 ± 0.12	8.25 ± 1.14	5.12 ± 1.67
IL-2 + MPA	0.15 ± 0.04	0.30 ± 0.03	0.16 ± 0.02
IL-2 + MZB	ND	0.33 ± 0.09	0.59 ± 0.05
IL-2 + RPM	0.85 ± 0.16	0.98 ± 0.13	1.31 ± 0.28
IL-15	0.38 ± 0.01	6.85 ± 0.53	5 ± 0.9
IL-15 + MPA	0.01 ± 0	0.05 ± 0.01	0.06 ± 0.01
IL-15 + MZB	ND	1.19 ± 0.13	0.90 ± 0.04
IL-15 + RPM	0.05 ± 0.01	0.73 ± 0.07	1.28 ± 0.09
IL-4	0.07 ± 0.01	0.1 ± 0.01	0.17 ± 0.01
IL-7	0.08 ± 0.01	0.53 ± 0.04	1.04 ± 0.02

<sup>a</sup> PBL were stimulated for 3 days with PHA (5 μg/ml) and 7 days with IL-2 (50 U/ml). Then cells were incubated with medium alone or with IL-2 (50 U/ml), IL-15 (10 ng/ml), IL-4 (500 U/ml), or IL-7 (20 ng/ml), in the presence or absence of MPA (10 μM), MZB (10 μM), or RPM (500 nM). [<sup>3</sup>H]TdR incorporation was evaluated as described in *Materials and Methods* and values are the mean ± SEM of three individual experiments.

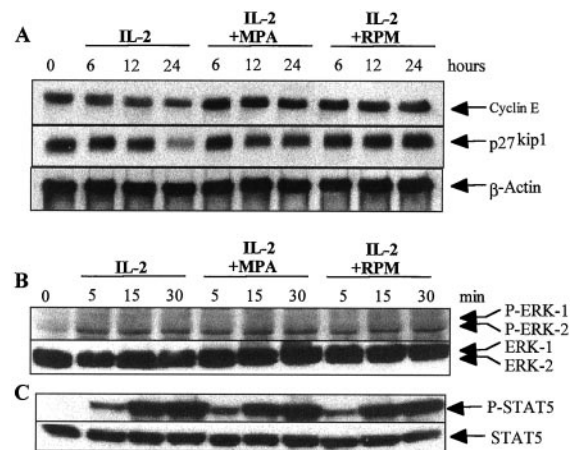
signaling, prevent the down-regulation of cFLIP protein levels and protect cells from CD95-mediated cell death (35). We first demonstrated that CD95 up-regulation following T cell mitogenic activation was not modified by MPA (data not shown). We then investigated the effect of MPA on acquisition of sensitivity to CD95-mediated apoptosis. We measured anti-CD95-induced cell death of PBL activated for 3 or 5 days with PHA in the presence of MPA or RPM. As previously reported, the presence of RPM during T cell activation inhibited anti-CD95-induced cell death (34, 35). Interestingly, MPA did not inhibit apoptosis induced by anti-CD95 mAb (Fig. 7A). In agreement with these observations, RPM, but not MPA, inhibited the down-regulation of cFLIP induced by PHA stimulation at 72 h (Fig. 7B).

#### Discussion

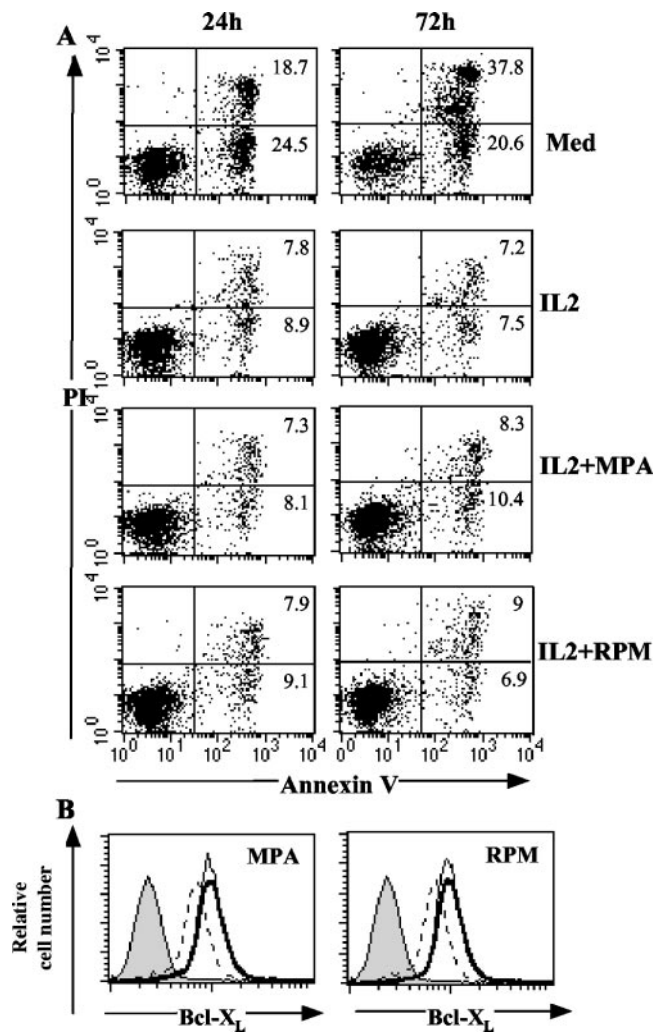
The immunosuppressive activity of MMF is usually attributed to its antiproliferative effect on T and B lymphocytes (8). Indeed its



**FIGURE 4.** Effect of MPA on cell viability. PBL were activated with PHA (5 μg/ml) in the presence or absence of 10 μM MPA, 500 nM RPM, or 10 μg/ml ARIL-2. **A**, PBL were labeled by CFSE at the onset of culture as described in *Materials and Methods* and after 5 days of culture, cell divisions were analyzed by FACS. Viable cell numbers determined by trypan blue exclusion (**B**) and percentage of annexin V<sup>+</sup> cells (**C**) of cells incubated in the presence of medium alone (×), PHA (●), PHA + MPA (△), PHA + RPM (□), or PHA + ARIL-2 (◇) were measured at the indicated time. Values are mean ± SEM from a triplicate measurement of one experiment from a total of two showing similar results.

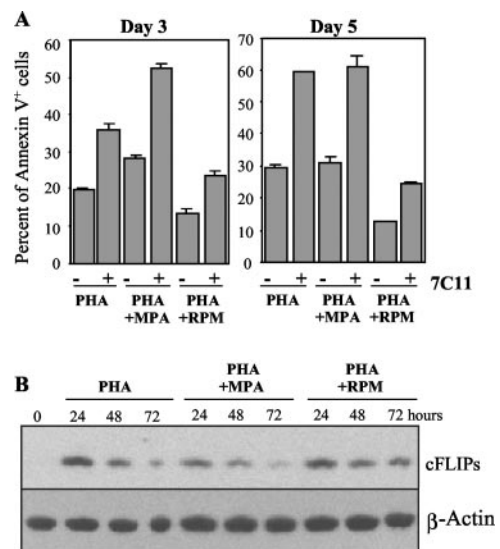


**FIGURE 5.** Effect of MPA and RPM on cyclin E, p27<sup>Kip1</sup> expression and ERK and STAT5 activation. PBL were activated for 3 days with PHA (5 μg/ml) then further incubated with IL-2 (50 U/ml) for 3 days. Cells were starved and cultured with MPA (10 μM) or RPM (500 nM) for 16 h. Then cells were re-stimulated with IL-2 (50 U/ml) for the indicated time. Expression of cyclin E and p27<sup>Kip1</sup> (**A**), phosphorylation of ERK1/ERK2 (**B**), and STAT5 (**C**) were evaluated by Western blot as described in *Materials and Methods*.



**FIGURE 6.** Effect of MPA and RPM on IL-2-dependent cell survival. Activated lymphoblasts were obtained as described in *Materials and Methods* and incubated alone or with IL-2 (50 U/ml), in the presence of MPA (10  $\mu$ M) or RPM (500 nM) for 24 or 72 h. *A*, At the indicated time, cells were collected and apoptotic cells were assessed. Results are representative of three experiments. *B*, After 24 h of culture, cells were collected and stained for Bcl-x<sub>L</sub>. Isotype-matched IgG was used as a control (shaded histogram). Results show Bcl-x<sub>L</sub> expression 24 h in the absence of IL-2 (dotted line), in the presence of IL-2 (thin line), or in the presence of IL-2 and drugs (bold line) (MPA, left panel; RPM, right panel). Histograms are representative of two independent experiments.

active metabolite, MPA, was shown to inhibit T cell proliferation induced by mitogens or allogeneic stimulator cells (7–9). This cytostatic effect was previously shown to result from a blockade in the early to mid-G<sub>1</sub> phase of the cell cycle (9). Consistent with these data, we show in this study that blockade in G<sub>1</sub> is not associated with a major drop in cell viability (Fig. 4). Furthermore it affects both CD4 and CD8 in parallel (Fig. 1*B*). We (Fig. 2), and others (8) demonstrated that MPA did not interfere with initial activation events following TCR engagement such as blast transformation or CD69 and CD25 expression. Expression of CD71 and CD154 was not affected at day 1, but later up-regulation of these markers as a consequence of cell proliferation was suppressed. Among immunosuppressive agents that block T cell proliferation, the calcineurin inhibitors cyclosporin A and FK506 inhibit IL-2 gene expression, whereas CD25 mAbs interfere with IL-2 binding to its receptor and RPM with IL-2R signaling (36). Because IMPDH inhibitors like MZB (14) and MPA did not decrease IL-2 synthesis



**FIGURE 7.** Effect of MPA and RPM on acquisition of sensitivity to CD95-mediated apoptosis. *A*, After 3 (left panel) or 5 (right panel) days of culture in the presence of PHA alone, or with MPA (10  $\mu$ M) or RPM (500 nM), activated PBL were incubated in 96-well microplates alone or in the presence of the anti-CD95 mAb 7C11 (1  $\mu$ g/ml). After 16 h, cell death was evaluated as described in *Materials and Methods*. Values are the mean  $\pm$  SEM of a triplicate experiment, representative of three individual experiments. *B*, Down-regulation of FLIP induced by PHA stimulation alone or in presence of MPA or RPM was measured by Western blot as described in *Materials and Methods*.

and IL-2R expression, one may postulate that, like RPM, they could interfere with IL-2R, but not TCR, signaling. Mitogenic activation by lectins or CD3 and CD28 mAbs combine TCR and IL-2R signaling. To study IL-2R signaling in the absence of associated TCR triggering, we used preactivated T cells cultured in IL-2-supplemented medium. Those cells are in G<sub>1</sub> and can be induced to proceed to S phase by addition of IL-2 or IL-15 without further TCR stimulation. In this model, we showed that MPA inhibits cytokine-stimulated proliferation. Since this effect was reversed by Gua and shared with MZB, it can be attributed to IMPDH blockade.

Laliberté et al. (9) attributed the antiproliferative effect of MPA to the inhibition of 1) cyclin D/CDK6 induction and 2) down-regulation of the CDK inhibitor p27<sup>Kip1</sup> following PHA stimulation of PBL. p27<sup>Kip1</sup> is a member of a family of CDK inhibitors that also includes p21<sup>Cip1/Waf1</sup> and p57<sup>Kip2</sup> (27). It binds to both CDK2 and CDK6 and therefore controls the activity of cyclin D/CDK6 and cyclin E/CDK2 complexes. In T cells, IL-2 was shown to down-regulate p27<sup>Kip1</sup> gene expression (12, 13). However, in vitro, p27<sup>Kip1</sup> protein was demonstrated to be not only an inhibitor, but also a substrate of the cyclin E/CDK2 complex, depending on the ATP concentration (37). Indeed, at low ATP concentration, p27<sup>Kip1</sup> interacts with the cyclin E/CDK2 complex and maintains the enzyme in a catalytically inactive form, whereas at a higher concentration, CDK2 binds ATP and can phosphorylate p27<sup>Kip1</sup> which triggers its ubiquitin-dependent degradation (37). Similarly to what was previously reported for RPM (13), we demonstrated in this study that MPA inhibits IL-2-induced down-regulation of p27<sup>Kip1</sup> (Fig. 5*A*). Thus IL-2-dependent regulation of p27<sup>Kip1</sup> appears as a common feature of MPA and RPM, two immunosuppressive drugs which specifically inhibit G<sub>1</sub>- to S-phase progression. However, whether MPA and RPM control p27<sup>Kip1</sup> down-regulation by the same or a distinct mechanism remains to be demonstrated. A recent study from Qiu et al. (6), using primary



lymphocytes demonstrated that MPA not only affects GTP but also ATP synthesis as an indirect consequence of IMPDH inhibition and GTP depletion. Indeed GTP is a cofactor for adenylysuccinate synthetase, the enzyme that catalyses the first step of AMP synthesis from inosine monophosphate. Whether reduction of ATP pools by MPA can directly affect activity of the cyclin E/CDK2 complex and p27<sup>Kip1</sup> phosphorylation is an appealing hypothesis that deserves further experiments.

IL-2R initiates multiple signaling pathways controlling not only proliferation, but also survival of activated T lymphocytes as well as their acquisition of susceptibility to CD95-mediated cell death (34, 38). We demonstrate in this study that the effect of MPA is restricted to the control of proliferation. Working with activated lymphoblasts rather than naive PBL offers the advantage of studying the effect of MPA on cytokine-dependent events without further TCR stimulation. As already mentioned, those activated lymphoblasts are dependent on IL-2 or IL-15 for their proliferation (Table I) whereas their survival is supported by either IL-2, -4, -7, or -15 (Fig. 6 and data not shown), which all signal through the IL-2R common  $\gamma$ -chain. We show in this study that while blocking IL-2-stimulated proliferation (Table I), not only MPA, but also RPM preserved IL-2-driven T cell survival (Fig. 6A). In keeping with those results, we observed that MPA does not affect up-regulation of Bcl-x<sub>L</sub> expression by cytokines. Indeed survival signals mediated by cytokines were demonstrated to require the common  $\gamma$ -chain of their receptor (15) and to correlate with the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x<sub>L</sub> (16).

Activated, but not naive, T cells are susceptible to CD95-mediated apoptosis (23, 39). The molecular basis for this difference relies on the ability of IL-2 to suppress transcription and expression of cFLIP. cFLIP competitively inhibits binding of caspase-8 to CD95 receptor, thus shutting off the downstream CD95 signaling pathway (40, 41). In agreement with those observations, inhibition of IL-2 production by cyclosporin A or IL-2 signaling by anti-IL-2 neutralizing mAbs or by RPM was shown to block susceptibility to CD95-mediated apoptosis of human activated PBL by preventing cFLIP degradation (35). Interestingly, we demonstrate in this study that MPA does not prevent activation-induced cFLIP degradation and priming for CD95-mediated apoptosis (Fig. 7). These data demonstrate that G<sub>1</sub>-blocked T cells in the presence of IL-2 are sensitive to CD95-mediated apoptosis. Therefore, these data reinforce the previous demonstration that T cells require IL-2, but not G<sub>1</sub>- to S-phase, transition to acquire susceptibility to CD95-mediated apoptosis (34, 38) and extend this observation to cFLIP down-regulation. Of note, priming of human PBL to CD95-mediated cell death was inhibited by RPM (our data and Ref. 35), whereas that of mouse splenocytes was not affected (24).

In organ transplantation, immunosuppressive treatments should not only prevent rejection but also, whenever possible, favor the development of active tolerance allowing the reduction of maintenance therapy. Interference with the "second signal" of T cell activation by blocking CD154 (CD154 mAbs) or CD28 (CTLA4-Ig) was shown to induce tolerance to allografts in nonhuman primates (42, 43). However, in such models, calcineurin inhibitors may antagonize tolerance induction (22), in keeping with the well-documented requirement for IL-2 in several models of natural tolerance or acquired tolerance to allografts (17, 18, 24). Hence, the lack of interference of MPA with IL-2 synthesis and IL-2-dependent sensitivity to activation-induced cell death could represent an advantage of MMF over calcineurin inhibitors. Several mechanisms could operate in IL-2-dependent tolerance, including sensitivity to activation-induced cell death induced by CD95-dependent (19) or CD95-independent (44) pathways, and also the function of regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells (45, 46). Those regulatory T cells

do not produce IL-2 but are totally dependent on exogenous IL-2 for growth and survival. They are missing in IL-2- and IL-2R $\alpha$ -deficient mice (20, 21). Gregori et al. (47) recently reported that short treatment of mice with the association 1 $\alpha$ ,25 dihydroxyvitamin D<sub>3</sub> and MMF induces tolerance to islet allografts and is associated with an increased frequency of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, suggesting that MMF do not prevent regulatory T cell expansion.

In conclusion, we demonstrate that MPA does not affect early activation events mediated by TCR engagement, but rather inhibits IL-2- or IL-15-driven proliferation of activated T cells, by interfering with down-regulation of p27<sup>Kip1</sup>. Furthermore, we demonstrate that other cytokine-dependent events, such as survival of activated T cells or IL-2 priming for cell death, are not affected by MPA. In this respect, MPA has unique functional properties not shared by other immunosuppressive drugs interfering with IL-2R signaling events such as RPM and CD25 mAbs. In the context of tolerance induction, such information may be considered in the design of new protocols with MMF.

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