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The Immunosuppressor Mycophenolic Acid Kills Activated Lymphocytes by Inducing a Nonclassical Actin-Dependent Necrotic Signal

Benjamin Chainge-Delalande,*§§ Gwendaline Guidicelli,*§§ Lionel Couzi,*†‡§ Pierre Merville,*‡ Walid Mahfouf,*‡ Stéphane Bouchet,*¶¶ Mathieu Molimard,*¶¶ Benoit Pinson,*# Jean-François Moreau,*†‡§ and Patrick Legembre3*†‡§

Mycophenolate mofetil (MMF) is an immunosuppressive agent used in transplantation. Over the last decade, MMF has also emerged as an alternative therapeutic regimen for autoimmune diseases, mainly for patients refractory to other therapies. The active compound of MMF, mycophenolic acid (MPA), depletes the intracellular pool of guanosine triphosphate through inosine monophosphate dehydrogenase blockade. The molecular mechanism involved in the elimination of T and B lymphocytes upon inhibition of inosine monophosphate dehydrogenase remains elusive. In this study, we showed that in contrast to the immunosuppressors azathioprine, cyclosporin A, and tacrolimus, MPA killed lymphocytes through the activation of a caspase-independent necrotic signal. Furthermore, the MPA-mediated necrotic signal relied on the transmission of a novel intracellular signal involving Rho-GTPase Cdc42 activity and actin polymerization. In addition to its medical interest, this study sheds light on a novel and atypical molecular mechanism leading to necrotic cell death. The Journal of Immunology, 2008, 181: 7630–7638.

Mycophenolic acid (MPA) is a prodrug that is metabolized into its active moiety termed mycophenolic acid (MPA) and is widely used to prevent rejection of kidney allografts in recipients (1, 2). MPA is a specific and non-competitive inhibitor of IMPDH. IMPDH inhibition drives a depletion of GTP that alters processes such as DNA replication, transcription, and protein translation and hence leads to a G1/S cell cycle arrest. In addition, the GTP depletion impairs the transfer of fucose and mannose to glycoproteins and reduces the expression of adhesion molecules, which are required for the attachment of leukocyte to endothelial cells (rolling lymphocytes) and the interaction between APCs and lymphocytes (Ag presentation) (for review see Ref. 3). An increase in IMPDH activity has been reported in solid neoplastic cells or in leukemic cells (4, 5), and the administration of IMPDH inhibitors protects patients from developing tumors (6, 7) by a yet undefined mechanism that may involve modulation of cell death (8, 9). Despite >3,000 publications investigating the functional and the molecular effects of MPA on cells, its cytotoxic action remains poorly defined. If activation of caspases is considered as essential to induce the MPA-mediated cell death signal, to date the molecular mechanism responsible for caspase activation upon MPA incubation is unknown (8–10).

Cell death has been divided into three pathways: programmed cell death, also termed apoptosis, autophagic cell death, and necrotic cell death (11). Because numerous markers exist to accurately define the activation status and the intensity of the apoptotic process, this signaling pathway has been extensively studied. Indeed, cleavage of a cysteine protease family-termed caspases (for review see Ref. 12) is an useful marker to easily define whether cells are dying from an apoptotic signal. Although necrosis has long been described as an unanticipated and inappropriate destruction of the cell (13), recent clues challenge this dogma (14, 15).

In the context of allogeneic organ transplant, down-regulation of the immune system is usually reached by using a combination of two immunosuppressors such as azathioprine or MMF associated with cyclosporin A or tacrolimus. Cyclosporin A and tacrolimus are potent inhibitors of the phosphatase calcineurin (protein phosphatase 2B) (16), which prevents the activation of NF-AT an essential step in lymphocyte activation, proliferation, and synthesis of the cytokine IL-2. Azathioprine and its active compound, 6-mercaptopurine, block lymphocyte proliferation by acting on still poorly defined molecular targets that disturb the purine synthesis (17).

In this study we showed that specific inhibition of the IMPDH mediates elimination of lymphocytes, basically through...
FIGURE 1. MPA induces a caspase-independent cell death signal through inhibition of IMPDH activity. A, The de novo synthesis of guanosine tri-phosphate. The salvage pathway is depicted in gray. IMP, Inosine monophosphate; XMP, xanthine monophosphate; HPRT, hypoxanthine-guanine phosphoribosyltransferase. B, Azathioprine and MPA-mediated cell death was assessed using a MTT viability assay. The T cell lines (Jurkat, H9) and the B cell lines (SKW6.4, Dab-1) were pretreated with or without caspase inhibitor zVAD-fmk (50 μM) for 30 min and the cells were then stimulated with the indicated dose of azathioprine or MPA for 48 h. C, The T cell line Jurkat and the B cell line Dab-1 were preincubated in presence of zVAD-fmk (50 μM) or DMSO (control) for 30 min and the cells were then stimulated with the indicated dose of ribavirin for 48 h. Total cell death was measured by MTT. D, The Jurkat T cell line was preincubated for 30 min with the indicated concentration of guanosine or adenosine and then treated with MPA (3 μg/ml) for 48 h. Cell death was measured using the MTT assay. Results shown represent the mean ± SD of three independent experiments.

Materials and Methods

Cell lines and PBLs

The T cell lines Jurkat, CEM, and H9 and the EBV-transformed B lymphoblastoid cells Dab-1 and SKW6.4 were grown in RPMI 1640 supplemented with 8% FCS. Fas-associated death domain protein (FADD)-deficient Jurkat (T2.1) and A3 control cells were purchased from American Type Culture Collection. The SVT35 and the receptor-interacting protein (RIP)-deficient Jurkat cells were provided by Dr. B. Seed (Harvard Medical School, Boston, MA). The pEGFP-Bcl-2 plasmid was provided by Dr. R. Youle (National Institutes of Health, Bethesda, MD). The generation of stable clones overexpressing Bcl-2 cells is described elsewhere (18). PBLs from healthy donors were isolated and stimulated as previously described (19).

Cells (5 × 10⁶ cells in 0.3 ml) were electroporated with 10 μg of DNA using a BTM 830 electroporation generator (BTX Instrument Division). Twenty-four hours later, living cells were isolated using the Ficoll separation method.

Reagents

Mycohenolic acid, azathioprine, and DiOC6 were purchased from Sigma-Aldrich. Homemade soluble FasL was generated in the laboratory (20). N-benzylxocarboxyl valine-alanine-aspartate-fluoromethylketone (zVAD-fmk), cytochlasin D, latrunculin A, and Clostridium difficile toxin B were purchased from Calbiochem. The plasmids containing the GTPase mutants N17-Cdc42, N17-Rac1, and N19-RhoA were obtained from Dr. P. Fort (Centre de Recherche de Biochimie Macromoléculaire, Montpellier, France). The anti-human caspase-3 mAb was purchased from BD Pharmingen. The anti-human caspase-8 (C-15) mAb was purchased from Axxora. The anti-microtubule-associated protein (MAP) L chain 3 (LC3) mAb was from Affinity Bioreagents, anti-Cdc42 was from BD Biosciences, anti-Rac1 was from Upstate Biotechnology, and anti-RhoA was from Santa Cruz Biotechnology. The anti-β-actin mAb was from Sigma-Aldrich.

Detergent lysis experiments and Western blot analysis

Cells were lysed for 30 min at 4°C in lysis buffer (25 mM HEPES (pH 7.4), 1% Triton X-100, and 150 mM NaCl) supplemented with a mix of protease inhibitors (Sigma-Aldrich). Protein concentration was determined using the bicinchoninic acid method (Sigma-Aldrich) according to the manufacturer’s protocol. Proteins were separated by SDS-PAGE on a 12% gel in reducing conditions and transferred to a nitrocellulose membrane (Amerham Biosciences). The membrane was blocked 1 h with TBST (50 mM Tris, 160 mM NaCl, 0.1% Tween 20 (pH 7.8)) containing 5% dried skimmed milk, and all subsequent steps were performed in this buffer. Specific primary Ab was incubated overnight at 4°C. After intensive washes, the secondary peroxidase-labeled Ab was added for 1 h and the proteins were visualized with the ECL substrate kit (Amersham). Cell death assays

Quantification of fragmented DNA (sub-G1 population) was performed as described elsewhere (21). Briefly, treated or untreated cells (0.2 × 10⁶) were harvested and incubated for 4 h in 300 μl of buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide (Sigma-Aldrich). Cell fluorescence was analyzed by flow cytometry. Cells exhibiting an intensity of fluorescence lower than the G1-G0 peak were counted as DNA-fragmented cells.

Total cell death was assessed using MTT assay (metabolic assay), as described previously (22). To measure cell permeability (damage of the plasma membrane), treated or untreated cells were incubated for 1 h with medium supplemented with 20 μg/ml propidium iodide and immediately analyzed by flow cytometry. For measurement of mitochondrial potential (Δψm), cells were preincubated with DiOC6 (10 nM) for 15 min and then stimulated with FasL or MPA. Cell fluorescence was measured by flow cytometry.

RhoA, Rac1, and Cdc42 activity assays

GST-PAK-CD and GST-C21 were provided by Dr. J. G. Collard (Netherlands Cancer Institute, Amsterdam, Netherlands). Cloning of the GST-PAK-CD fusion protein containing the Rac and Cdc42 binding region from

RhoA, Rac1, and Cdc42 activity assays

GST-PAK-CD and GST-C21 were provided by Dr. J. G. Collard (Netherlands Cancer Institute, Amsterdam, Netherlands). Cloning of the GST-PAK-CD fusion protein containing the Rac and Cdc42 binding region from
three independent experiments.

GTPase activity assays were conducted as previously described (23). In

human PAK1B, has previously been described (23). GST-C21 has been

GTPase activity assays were conducted as previously described (23). In

brief, cells were treated or untreated for the indicated times and lysed for

30 min in a lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5%}

sodium deoxycholate, and 1% Triton X-100, supplemented with mixtures of

protease and phosphatase inhibitors) effective for solubilizing the plasma

membrane-distributed small GTPases (23). The cell lysates were next

incubated for 2 h with GST-PACK-CD or GST-C21 bound to glutathione-
coupled Sepharose beads. Beads were washed in lysis buffer and resus-
pended in Laemml sample buffer. The amount of bound Cdc42, Rac1, or

RhoA molecules was analyzed by Western blotting.

Flow cytometry

Cells were washed in PBS with 1% (w/v) BSA, and fluorescence was

immediately analyzed using a FACScalibur flow cytometer (BD Bioscience).

Immunofluorescence and imaging

Cells were let to adhere for 5 min at room temperature to poly-tlysine-

coated slides (ESCO, VWR). Cells were fixed in PBS with 2% PFA for 30

min, washed, and then treated for 15 min with PBS containing 5% FCS to

quench aldehyde groups. Cells were permeabilized for 5 min at 4°C using

PBS and 0.1% Triton X-100 and stained with tetramethylrhodamine iso-
thiocyanate-linked phalloidin (Sigma-Aldrich) (1 µg/ml) for 30 min at

4°C. Slides were washed, dried, and mounted with fluorescent mounting

medium (DakoCytomation). Images were analyzed with a confocal micro-

scope (Leica SP5) with a ×63 objective.

Electronic microscopy

Stimulated cells (10^7) were fixed for 1 h at 4°C in 1.5% glutaraldehyde (0.1M

phosphate buffer), postfixed in 1% osmium tetroxide for 1 h at 4°C, dehydrated

in ethanol, and embedded into a Epon resin mixture. Sections were stained

using uranyl acetate and lead citrate and sections were analyzed using a FEI

Tecnai 12 BioTwin transmission electron microscope. Pictures were acquired

with a Gatan Orius wide-angle camera (11 megapixels).

Results

MPA induces a caspase-independent cell death signal

T and B leukemia cell lines were incubated with therapeutic concen-

trations of azathioprine, MPA, cyclosporin A, or tacrolimus. Whereas cells were

efficiently eliminated upon MPA and azathioprine treat-

ments (except for the azathioprine-resistant T lymphoma cell line H9; Fig.

1B), cyclosporin A and tacrolimus either exerted a faint cytotoxic

effect or were devoid of one (data not shown). As caspase activity is

the landmark of the apoptotic signal, we next determined the role of

these proteases in the MPA and azathioprine-mediated cell death sig-

nals. Strikingly, whereas preincubation of cells with the pan-caspase

inhibitor zVAD-fmk prevented cell death mediated by azathioprine

treatment (Fig. 1B), it was inoperative on the cytotoxic effect of MPA

(Fig. 1B). Identical results were obtained using the executioner
caspase inhibitor DEVD-chloromethyl ketone (data not shown). To

confirm that this caspase-independent signal relied on the inhibition of

IMPDH, we treated the cells with a competitive inhibitor of IMPDH

called ribavirin (25). Identically to MPA, ribavirin induced a caspase-

independent cell death signal in T or B cell lines (Fig. 1C). These

findings indicated that in contrast to other immunosuppressive agents,

MPA generated a caspase-independent cell death in T and B cell lines

through the inhibition of IMPDH.

Inhibition of IMPDH leads to a decrease of the intracellular pool

of GTP (26), which can be counterbalanced by the addition of

exogenous guanosine (Fig. 1A, see “Salvage pathway”). To dem-

onstrate that MPA-mediated cell death was specifically due to the

abrogation of IMPDH activity, rescue of cells by the addition of

guanosine was analyzed. Adenosine acts on the ATP pool and was

used as a control. Although adenosine did not modify the MPA-

mediated cell death, guanosine abrogated the cytotoxic effect of

MPA (Fig. 1D) indicating that cell death was exclusively mediated

through the inhibition of IMPDH.

MPA kills cells mainly through a caspase-independent cell death

It was conceivable that MPA-mediated depletion of GTP could

account for a general decrease of the metabolic activity in living

FIGURE 2. MPA-induced cell death is caspase independent. A, Mitochon-
dria depolarization was assessed using DiOC6 staining. The T cell line

Jurkat and the B cell line Dab-1 were treated with or without zVAD-fmk

(zVAD, 50 µM) and next stimulated with MPA (3 µg/ml) for the indicated

times. In the upper panel are depicted histograms of DiOC6 staining ob-
tained with Jurkat cells preincubated for 30 min in presence of zVAD-fmk

(50 µM) or DMSO (control) and then untreated or treated for 6 h with the

apoptotic inducer FasL (100 ng/ml). In the lower panel, cells were incu-
bated for 30 min with 50 µM zVAD-fmk or DMSO and DiOC6 fluores-
cence was analyzed using flow cytometry. The percentage of cells that
underwent a loss of mitochondria depolarization was assessed using DiOC6 staining. The T cell line

Jurkat and the B cell line Dab-1 were treated with or without zVAD-fmk

(zVAD; 50 µM) for 30 min before stimulation with

FasL (100 ng/ml). Influx of propidium iodide was analyzed by flow cytometry and the

percentage of fluorescent cells was plotted.

B, T (Jurkat) and B

(Dab-1) cell lines were treated as described above and then incubated for

1 h with the fluorescent DNA intercalating agent propidium iodide (20 µg/ml). Influx of propidium iodide was analyzed by flow cytometry and the

percentage of fluorescent cells was plotted. C, Indicated cells were treated with or without zVAD-fmk (50 µM) for 30 min before stimulation with

MPA (3 µg/ml) for 48 h. DNA fragmentation (sub-G1 cell population) was
determined using flow cytometry analysis as described in Materials and

Methods. Lower panel, The results shown represent the mean ± SD of

three independent experiments. D, Jurkat cells were preincubated for 30

min with zVAD-fmk (50 µM) or DMSO and then treated or untreated with

MPA (3 µg/ml) for 48 h. Cells were lysed and caspase-8 (initiator) or

caspase-3 (executioner) activities were quantified over time by adding Ile-

Glu-Thr-Asp (IETD)-pNA or Asp-Glu-Val-Asp (DEVD)-pNA, respect-

ively. Data are representative of five independently performed

experiments.
cells and that the MTT assay measuring the mitochondrial and cytoplasmic dehydrogenase activities was not a faithful indicator of cell death. To rule out this possibility, MPA-mediated cell death was assessed using several methods quantifying the loss of mitochondrial potential (ΔΨm), DNA fragmentation (apoptosis), morphological changes, and plasma membrane damage (propidium iodide influx). Engagement of the apoptotic receptor Fas (APO1/CD95) led to complete mitochondria depolarization, and inhibition of the caspase activity reverted the loss of ΔΨm (Fig. 2A, upper panel). In contrast, MPA treatment induced the depolarization of the mitochondria independently of the caspase activity (Fig. 2A, lower panel). MPA also induced damages of the plasma membrane that were not prevented by the use of zVAD-fmk (Fig. 2B). Surprisingly, a small part of the MPA-treated cells harbored fragmented DNA, a hallmark of apoptosis, and these cells were protected from death by zVAD-fmk preincubation (Fig. 2C). It was confirmed that some cells were dying through an apoptotic signal, because caspase-8 and caspase-3 activities were detected in the whole lysates of MPA-treated cells (Fig. 2D). These findings pointed out that upon MPA treatment, a major part of the cells died through a caspase-independent signal while a minor part of the cells were eliminated by a caspase-dependent apoptotic signal.

Inhibition of caspase activity did not impinge on total MPA-mediated cell death (Figs. 1B and 2, A and B). One possibility to explain this discrepancy was that the caspase inhibitor zVAD-fmk did not fully inhibit the catalytic activity of these proteases. Using caspase activity assays, we ruled out this possibility because the pan-caspase inhibitor totally abrogated the caspase activities in MPA-treated cells (Fig. 2D).

**Caspase-independent cell death exhibits necrotic features**

Flow cytometric parameters, cell size (forward scatter) and granule content of the cells (side scatter), allowed us to define a window corresponding to the living cells (untreated cells; see black gate in Fig. 3A). Based on the cell population that died upon treatment with the apoptotic inducer FasL, a second window was defined corresponding to the apoptotic morphology (gate 2 in the pictogram, Fig. 3A). This cell population was a minority (between 10 and 30%) and died through a caspase-dependent signal because zVAD-fmk preincubation dramatically decreased the percentage of the cells present within this gate (see histogram, Fig. 3A). MPA treatment revealed a third population that appeared rapidly (24 h), comprised a majority of cells, and harbored a size similar to that of living cells but contained more granules (gate 3). Cells encompassed within the gate 3 were insensitive to the zVAD-fmk pretreatment (see Fig. 3, A and B). Strikingly, the decrease in the number of apoptotic cells due to caspase inhibition was counterbalanced by the appearance of a fourth population corresponding to small and highly granular cells (gate 4) (see histogram in Fig. 3A).

To identify the types of cell death induced upon MPA treatment, we used transmission electron microscopy. The T leukemia cell line Jurkat harbored a large nucleus containing one or more nucleoli, and the cytoplasm was homogeneous and devoid of any vacuoles (Fig. 4A). FasL stimulation led to a drastic modification of the cellular shape with the cytoplasmic accumulation of vacuoles and the condensation of chromatin around the nuclear periphery (Fig. 4A), a morphology typical of apoptotic cells (27). In contrast, following treatment with MPA a majority of cells (70 to 90%) displayed a loss of their plasma membrane integrity, mitochondria swelling, and, more strikingly, no trace of chromatin condensation (Fig. 4A). These morphological features are hallmarks of cells dying through a necrotic process (27). Ten to 30% of the cells treated with MPA displayed apoptotic features (data not shown) and the zVAD-fmk pretreatment eliminated this minor population, which was replaced by small sized cells exhibiting a cytoplasm containing numerous granules (Figs. 4B and 3A). Using higher magnification, these cells displayed accumulation of double-membrane vacuoles containing degraded organelles (see filled arrowheads; Fig. 4B), a hallmark of autophagic cells. Two forms of microtubule-associated protein 1 LC3 exist, a cytosolic LC3-I and an autophagosome membrane-bound LC3-II considered as a marker of the autophagic process (28). The autophagic population was confirmed in B and T cell lines, because the amount of LC3-II significantly increased when cells were preincubated with zVAD-fmk and treated with MPA (Fig. 4C).

In summary, MPA kills T and B lymphoma cell lines by inducing a necrotic morphology. For unknown reasons, a minority of the cells did not undergo the necrotic process but instead were eliminated via a caspase-dependent apoptotic signal. Upon inhibition of caspases, the cells protected from apoptosis underwent death through an autophagic process (see Figs. 3A and 4, B and C).
Activated T lymphocytes are killed through a necrotic signal

To determine whether MPA induced a necrotic signal in primary T lymphocytes, PBLs were harvested from healthy donors, activated, and then incubated with MPA (Fig. 5A). Activated lymphocytes were efficiently killed by MPA or ribavirin and both induced a cell death signal independent of the caspase activity (Fig. 5, A and B).

In contrast, azathioprine eliminated the PBLs through a caspase-dependent cell death signal (Fig. 5A). Analysis of the DNA fragmentation revealed that similar to data obtained with the cell lines, less than one-third of the MPA-killed T lymphocytes underwent apoptosis (Fig. 5C). It was noteworthy that naive PBLs were refractory to MPA-mediated cell death, whereas the activation process of T lymphocytes led to the sensitization of the cells to the MPA-mediated necrotic signal (Fig. 5C). Cell morphology analysis using transmission electron microscopy confirmed that inhibition of IMPDH either using MPA or ribavirin triggered necrosis of the activated PBLs (Fig. 5D).
MPA mediates a rapid and efficient necrotic signal

Necrosis is currently considered to be an uncontrolled mechanism that is observed when high doses of cytotoxic agents are used and/or the apoptosis process is abrogated (14). To determine whether the MPA-mediated necrotic signal occurred because the apoptotic pathway was altered, we analyzed the kinetics of necrotic and apoptotic cell death by measuring simultaneously DNA fragmentation (apoptosis) and morphological cell death (necrosis) as described above. The appearance of apoptotic cells was dramatically delayed compared with that of the necrotic cells (Fig. 6A). Indeed, whereas 10–20 h of incubation with MPA were sufficient to eliminate 20% of the cells via a necrotic process, the incubation had to be prolonged from 35 to 55 h to reach a similar percentage of DNA-fragmented cells (Fig. 6A). Because necrosis has been reported to occur when elevated concentrations of agent are used (14), we conducted a dose-effect analysis of MPA on B and T cell lines. We observed that whereas low doses of MPA between 0.1 and 0.4 μg/ml were sufficient to reach 20 to 25% of necrotic cells insensitive to zVAD-fmk pretreatment (morphologic cell death in Fig. 6B), 1 μg/ml MPA was necessary to quantify the same amount of caspase-dependent apoptotic cells (DNA fragmented cells in Fig. 6B). In conclusion, low doses of MPA induced a rapid and efficient necrotic cell death signal that took place before the induction of a delayed apoptotic signal requiring elevated concentrations of MPA.

MPA-mediated necrotic signal is independent of FADD and RIP

It has been reported that high doses of FasL induce a necrotic pathway that is amplified by both translation and transcription inhibitors (14). Hence, we examined the effect of de novo translation or transcription inhibition on the MPA-mediated necrotic signal. Strikingly, cycloheximide or actinomycin D incubations dramatically inhibited the MPA-mediated necrotic signal (Fig. 7A). Because the adaptor protein FADD and the serine-threonine kinase RIP have been reported as being essential for the transmission of a necrotic signal in T lymphocytes (14), we next examined the putative role of these proteins in the MPA signal. The RIP- and the FADD-deficient Jurkat T cell lines and their respective parental cell lines were incubated with MPA for 48 h. Cell death was assessed by MTT. C. Jurkat cells expressing GFP or the chimera protein GFP-Bcl2 were treated with or without MPA for 48 h and total dead cells (metabolic assay MTT) or apoptotic cells (sub-G1 cell population) were assessed. Expression of GFP-Bcl2 in each clone was estimated by immunoblotting. The indicated cells were lysed and 50 μg of protein was subjected to SDS-PAGE. Endogenous and GFP-fused Bcl2 were revealed using an anti-Bcl2 mAb. Results represent the mean ± SD of three independent experiments.

FIGURE 6. The necrotic signal requires short exposure with MPA and a low dose of MPA. A, A kinetic analysis was performed with a high concentration of MPA (3 μg/ml) on different T and B cell lines. Cell death was quantified using morphological (necrotic) or DNA fragmentation (apoptotic cell death) assays. B, Jurkat and Dab-1 cell lines were preincubated with or without 50 μM zVAD-fmk (zVAD) for 30 min and then treated for 48 h with the indicated concentration of MPA. Necrotic (morphology) and apoptotic (sub-G1 population) cells were quantified.

FIGURE 7. The necrotic signal does not rely on FADD or RIP expressions but requires protein synthesis. A, The B-lymphoblastoid Dab-1 cell line was preincubated for 1 h with actinomycin D (0.5 μg/ml) or cycloheximide (1 μg/ml) and then treated with MPA (3 μg/ml) for 24 h. Cell death was quantified using the MTT assay. B, Parental cells and FADD-deficient or RIP-deficient Jurkat cells were stimulated with MPA for 48 h. Cell death was assessed by MTT. C, Jurkat cells expressing GFP or the chimera protein GFP-Bcl2 were treated with or without MPA for 48 h and total dead cells (metabolic assay MTT) or apoptotic cells (sub-G1 cell population) were assessed. Expression of GFP-Bcl2 in each clone was estimated by immunoblotting. The indicated cells were lysed and 50 μg of protein was subjected to SDS-PAGE. Endogenous and GFP-fused Bcl2 were revealed using an anti-Bcl2 mAb. Results represent the mean ± SD of three independent experiments.
and N19-RhoA-GFP or GFP alone. To quantify the activity of RhoA, Cdc42, and Rac1, pull-down assays were conducted as described in Materials and Methods.Jurkat cells were transiently transfected with expression vectors encoding the dominant negative constructs N17-Cdc42-GFP, N17-Rac1-GFP, and N19-RhoA-GFP or GFP alone. Left panel, Transfected cells were incubated with MPA (3 μg/ml) and the viable cells were quantified using morphology analysis (flow cytometry). Right panel, the transfected cells were stimulated with MPA and the ratio of GFP-positive cells to living cells was quantified following image acquisition and processing with a confocal microscope (Leica SP5) using a 63 objective. White arrowheads indicate the barely detectable phalloidin staining of the dominant negative Cdc42 (N17-Cdc42)-expressing cells. The relative amount of polymerized actin was assessed by densitometry (ImageJ analysis) in quantifying the intensity of phalloidin fluorescence (area of 0.5 μm²) under the plasma membrane of indicated cells. Independent counts (46 counts) were performed and the mean of fluorescence (±SD) was depicted. Statistical analysis was conducted using a Mann-Whitney test. 

Results represent the mean ± SD of three independent experiments. B, Jurkat cells were transiently transfected with expression vectors encoding the dominant negative constructs N17-Cdc42-GFP, N17-Rac1-GFP, and N19-RhoA-GFP or GFP alone. Left panel, Transfected cells were stimulated with MPA (3 μg/ml) and the viable cells were quantified using morphology analysis (flow cytometry). Right panel, the transfected cells were stimulated with MPA and the ratio of GFP-positive cells to living cells was quantified following image acquisition and processing with a confocal microscope (Leica SP5) using a 63 objective. White arrowheads indicate the barely detectable phalloidin staining of the dominant negative Cdc42 (N17-Cdc42)-expressing cells. The relative amount of polymerized actin was assessed by densitometry (ImageJ analysis) in quantifying the intensity of phalloidin fluorescence (area of 0.5 μm²) under the plasma membrane of indicated cells. Independent counts (46 counts) were performed and the mean of fluorescence (±SD) was depicted. Statistical analysis was conducted using a Mann-Whitney test. 

The necrotic signal requires Cdc42 activation and polymerized actin Recently, it has been shown that adenoviruses are able to activate members of the Rho-GTPases and thus induce an atypical cell death in cancer cells (30). Therefore, the expression level and the activity status of the principal Rho GTPases were analyzed upon incubation with MPA. Whereas the protein expression of RhoA and Rac1 remained constant during the MPA incubation (Fig. 8A), the MPA addition induced an increase of the Cdc42 protein level between 20 and 30 h (Fig. 8A). Using Rac1/Cdc42 and RhoA-specific effectors fused to GST in a pull-down assay (23), we quantified the amount of active GTPases during MPA treatment. Despite large amounts of RhoA in the cells, its active form was barely detectable and its activation level remained weak during MPA treatment (Fig. 8A). In contrast, Rac1 and Cdc42 were activated after 20 h of contact with MPA and remained active up to 44 h (Fig. 8A). Next, Jurkat and Dab-1 cell lines were transfected with dominant negative variants of the different Rho GTPases fused to the fluorescent protein GFP. Transiently transfected cells (20–30% of GFP-containing cells) were incubated with MPA and the percentage of living cells and the enrichment of GFP-expressing cells in this population were quantified (Fig. 8B). Strikingly, the ectopic expression of a dominant negative Cdc42 (N17-Cdc42) significantly protected the cells from MPA-mediated cell death (Fig. 8B). Furthermore, the ratio of green cells (GFP-positive cells) expressing the mutated Cdc42 (N17-Cdc42) was dramatically increased inside the living cell population (Fig. 8B). In contrast, transfection of GFP alone or RhoA and Rac1 dominant negative constructs neither modified the percentage of living cells (Fig. 8B, left panel) nor the ratio of GFP-positive cells upon MPA incubation (Fig. 8B, right panel). The B lymphoblastoid cell line Dab-1 was similarly protected from MPA necrotic signal upon N17-cdc42 expression (data not shown). C. difficile toxin B (toxin B) inhibits the biological activity of the Rho-GTPases (31). To validate that the activity of Rho-GTPases was essential to transmit necrosis in activated PBLs, we
incubated lymphocytes with toxin B. As can be observed in Fig. 8C; toxin B dramatically altered the MPA-mediated necrotic signal. These data indicated that Cdc42 was involved in the transmission of the necrotic signal.

Because Cdc42 is a key regulator of essential cellular functions such as cytoskeletal dynamics (32), we next evaluated whether the effect of Cdc42 on necrosis could be associated with its action on the actin cytoskeleton. A polymerized form of actin (actin-F) was labeled with the cyclic peptide phallolidin (33). As observed by confocal microscopy, T cells exhibited polymerized actin around the periphery of the cell (Fig. 8D). Whereas GFP alone did not modify the pattern of the polymerized actin network, the dominant negative Cdc42 significantly impaired the phallolidin labeling (see white arrowheads and densitometry analysis in Fig. 8D). To determine whether the necrotic signal was dependent on the polymerized actin network, we next tested the effect of two inhibitors of actin polymerization (latrunculin A (LtnA) and cytochalasin D (CytD)) on MPA-mediated cell death. Using noncytotoxic doses of LtnA and CytD, we showed that inhibition of actin polymerization dramatically abrogated the MPA-mediated cell death (Fig. 8E). Although these drugs displayed a significant effect (60% of inhibition) to prevent MPA-mediated necrosis in activated PBLs (Fig. 8F), their actions were weaker than that observed on the B cell line. Nevertheless, when caspase activity was inhibited, both LtnA and CytD totally abrogated the cell death signal in activated PBLs (Fig. 8E). Taken together these data showed that the remodeling of polymerized actin played an essential function in the transmission of the caspase-independent signal induced by MPA.

**Discussion**

Using different approaches, we demonstrated that the widely used immunosuppressive agent MPA triggers a potent and rapid necrotic signal. This necrotic signal was uniquely triggered upon MPA treatment, because other currently used immunosuppressive agents are either poorly cytotoxic (cyclosporin A, tacrolimus) or do not respond to the MPA-mediated necrotic signal but rather is mediated by the immunosuppressive agent MPA triggers a potent and rapid necrotic signal. This necrotic signal was uniquely triggered upon MPA treatment, because other currently used immunosuppressive agents are either poorly cytotoxic (cyclosporin A, tacrolimus) or do not respond to the MPA-mediated necrotic signal but rather is mediated by the immunosuppressive agent MPA (9, 10, 34). Inhibition of the caspase activity abrogated the MPA-mediated cell death (Fig. 8E). This necrotic signal was uniquely triggered upon MPA treatment, because other currently used immunosuppressive agents are either poorly cytotoxic (cyclosporin A, tacrolimus) or do not respond to the MPA-mediated necrotic signal but rather is mediated by the immunosuppressive agent MPA.

Resistance to apoptosis improves the genetic instability of the cell (e.g., DNA-damage foci, aneuploidy, supernumerary centromere) (38) and is an essential step in tumorigenesis (39) allowing the cell to escape from immunosurveillance (40). High incidence of lymphomas and atypical malignancies are a hallmark of renal transplant recipients (41). Recently, MMF treatment has been shown to decrease the risk of incidence of malignancy as compared with a non-MMF treated cohort of patients, and the incidence of lymphomas was lower in the MMF-exposed group vs the MMF-unexposed group (0.53 vs 0.95%) (42). Because, in contrast to other immunosuppressive agents, MPA incubation drives a potent necrotic signal and the IMPDH-inhibitor is also able to trigger apoptosis or autophagy depending on the functionality of the caspases, MPA-mediated cell death could be an attractive explanation for the lower occurrence of cancer observed in the renal transplanted patient compared with patients treated with azathioprine.

The necrotic signal induced by MPA may also explain why this agent is efficient for treating autoimmune diseases such as systemic lupus erythematosus (SLE). Indeed, it has been reported that deficiency of phagocytic activity and accumulation of apoptotic bodies could favor the progression of SLE (43). Because MPA mainly eliminated Ag-stimulated cells (not naïve cells) through necrosis, we could conceive that the down-modulation of the immune activity associated with an identical or decreased amount of apoptotic bodies may account for the efficiency of MPA in treating SLE (44).
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Disclosures

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