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Induction of apoptosis and modulation of activation and effector function in T cells by immunosuppressive drugs

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SUMMARY

Immunosuppressive drugs (ISD) are used for the prevention and treatment of graft rejection, graft-*versus*-host-disease (GVHD) and autoimmune disorders. The precise mechanisms by which ISD interfere with T cell activation and effector function or delete antigen-specific T cells are defined only partially. We analysed commonly used ISD such as dexamethasone (DEX), mycophenolic acid (MPA), FK506, cyclosporin A (CsA), rapamycin (RAP), methotrexate (MTX) and cyclophosphamide (CP) for apoptosis-induction and modulation of activation and effector function in human peripheral T cells, cytotoxic T cell lines (CTL) and Jurkat T cells. Of all drugs tested only CP and MTX prevented antigen-specific proliferation of T cells and decreased cytotoxicity of alloantigen specific CTL lines by direct induction of apoptosis. MTX and CP also slightly increased activation-induced cell death (AICD) and CD95-sensitivity. In contrast, all other drugs tested did not induce T cell apoptosis, increase CD95-sensitivity or AICD. CsA and FK506 even prevented AICD by down-modulation of CD95L. DEX, MPA, CsA, FK506 and RAP inhibited activation of naive T cells, but were not able to block proliferation of activated T cells nor decrease cytotoxic capacity of CTL lines. These results show that ISD can be classified according to their action on apoptosis-induction and inhibition of proliferation and would favour a rational combination therapy to delete existing reactive T cells and prevent further T cell activation.

Keywords activation-induced cell death apoptosis cyclophosphamide immunosuppressive drugs methotrexate

INTRODUCTION

Immunosuppressive drugs (ISD) are used clinically in autoimmune disorders and transplantation of allogeneic bone marrow or solid organs to establish peripheral allograft tolerance, to prevent or to treat graft-*versus*-host-disease (GVHD), and to suppress autoreactive T cells. However, the global effect of ISD on the immune system predisposes patients to the development of infection and cancer [1] and may have deleterious long-term effects on graft function [2]. Ideally, the goal of immunosuppressive therapy would be the induction of long-lasting immunological tolerance by selective deletion of antigen-specific and autoaggressive lymphocytes.

The mechanisms by which different ISD interfere with T cell activation and proliferation are diverse and to some extent well defined. Glucocorticoids, cyclosporin A and FK506 inhibit cytokine synthesis by T cells by preventing the action of nuclear transcription factors [3,4]. In contrast, rapamycin (RAP) inhibits the ability of lymphocytes to proliferate in response to IL-2 by preventing the down-regulation of cell cycle inhibitors such as Kip-1 and thus blocks the transition from G1 into S phase [5]. Mycophenolic acid and methotrexate exert antiproliferative effects by interfering metabolically with DNA synthesis [6,7] and cyclophosphamide inhibits T cell proliferation by introducing DNA damage [8]. While ISD have been described to prevent the activation and expansion of resting T cells, it is not known whether they silence the action of activated T cells and turn them into a state of unresponsiveness (anergy) or whether they delete antigen-activated T cells and lead to long-lasting immunological tolerance.

Induction of apoptosis is a key mechanism to delete autoreactive T cells during thymic selection [9] and to eliminate activated T cells in the periphery during the termination of an immune response [10]. CD95, also known as APO-1 or Fas, is a member of the death receptor family [11] important for T cell homeostasis. Sensitivity of T cells towards CD95-mediated apoptosis develops during the course of an immune response. While resting T cells express low amounts of CD95 and its ligand (CD95L) on the cell surface, expression levels increase during stimulation. Short-term CD95 positive activated T cells, however, are still resistant to apoptosis and become sensitive to apoptosis only around day 6 of activation [12]. Upon repeated TCR triggering, activated T cells undergo apoptosis, a mechanism called

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activation-induced cell death (AICD) which is mainly mediated via the CD95/CD95L system [13]. AICD plays a critical role in the termination of the immune response and induction of peripheral tolerance to self-antigens [14]. Mutations in CD95 or CD95L result in deficient AICD responses and lead to autoimmunity and lymphoproliferative-like diseases [15,16].

The effects of ISD on T cell apoptosis, CD95-sensitivity and AICD are not well known. Glucocorticoids have been shown to induce apoptosis of murine thymocytes [17], T cell hybridomas [18] and human leukaemic T cells [19]. Although methotrexate (MTX) was found to trigger CD95L expression in leukaemic T cell lines [20] and hepatoma cell lines [21], MTX may also induce apoptosis by a CD95-independent pathway [22]. Induction of CD95-dependent apoptosis by cyclophosphamide has been described for *in vivo* treatment of rat thymocytes [23]. Modulation of AICD by immunosuppressants has been found for glucocorticoids [24], CsA and FK506 [25]. These drugs, however, prevent up-regulation of CD95L after T cell activation and therefore inhibit AICD. In contrast RAP does not block AICD [26].

Here, we investigated whether commonly used ISD such as dexamethasone (DEX), mycophenolic acid (MPA), cyclosporin A (CsA), FK506, RAP, MTX, and cyclophosphamide (CP) interfere with apoptosis mechanisms in human leukaemic T cell lines and human peripheral T cells. Of all drugs tested only MTX and CP slightly increased AICD and CD95-sensitivity. Inhibition of proliferation in naive and activated T cells and reduction of T cell cytotoxicity by MTX and CP was caused by direct induction of apoptosis. None of the other drugs tested induced deletion of antigen-activated T cells but rather inhibited proliferation of naive T cells. These findings may be useful for the choice of immunosuppressants after organ transplantation or for the treatment of autoimmune diseases.

MATERIALS AND METHODS

Cell lines

All cell lines were grown in RPMI 1640 medium (GIBCO-BRL, Paisley, UK) supplemented with 10% heat-inactivated FCS (Biochrom KG, Berlin, Germany), 2 mM L-glutamine and 1 mM sodium pyruvate at 37°C in a humidified atmosphere containing 7·5% CO_2. Three HLA-A1$^-$ cell lines were used: the human LCL lines 721.221 [27] and C1R [28] and the mouse mastocytoma cell line P1.HTR [29]. The LCL line .721 expressed HLA-A1. For the establishment of P1.A1/hβ_2m, P1.HTR cells were co-transfected with genes encoding human HLA-A1 [30], human β_2m [31] and resistance to neomycin [32]. C1R.A1 transfectants were generated in the same way except that no human β_2m encoding plasmid was co-transfected. HLA-A1 surface expression was confirmed by flow cytometry using an A1 and A36 allelespecific antibody (BmT GmbH, Krefeld, Germany).

ISD

Dexamethasone (DEX), mycophenolic acid (MPA) and cyclosporin A (CsA) were obtained from Sigma, Germany, FK506 (Prograf®) from Fujisawa Pharmaceutical Co., Osaka, Japan and Rapamycin (RAP) from Calbiochem (Bad Soden, Germany). 4-Hydroperoxycylophosphamide (CP) was a gift from ASTA MEDICA, Frankfurt, Germany and methotrexate (MTX) from Wyeth Pharma, Wolfratshausen, Germany.

Induction and analysis of apoptosis

Jurkat cells (1×10^5/ml) were cultured in triplicate for 24 h on 96-well plates coated with OKT3 (100 μg/ml) (anti-CD3 ε-chain, obtained from American Type Culture Collection, Rockville, MD, USA). PBMC (1×10^6/ml) were activated with 100 ng/ml OKT3 and were restimulated after 6 days with 50 ng/ml PMA and 1 μg/ml ionomycin (Sigma, Germany) for 16 h. To determine CD95-sensitivity, cells were treated with anti Apo-1 IgG3 antibody [33]. Cell death was determined by quantification of DNA fragmentation by analysis of propidium iodide-stained nuclei [34], by measuring FSC/SSC or by Annexin V-FITC staining to externalized phosphatidylserine (Annexin V-FITC Kit, Bender Medical Systems, Vienna, Austria) on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany) and specific apoptosis was calculated according to the formula: $100 \times$ [experimental cell death (%) – spontaneous cell death (%)]/[100 – spontaneous cell death (%)]. Data always represent the mean of triplicates.

Lymphocyte isolation, biomagnetic separation and stimulation

PBMC were obtained from peripheral blood of healthy donors. Isolation was performed by density centrifugation of blood on Ficoll (Pharmacia Biotech AB, Uppsala, Sweden). CD4$^+$ and CD8$^+$ T cells were isolated from PBMC by depletion of monocytes by plastic adherence for 2 h at 37°C. Non-adherent cells were stained with supernatants from hybridomas A9 (anti-CD16, kindly provided by M. Pfreundschuh, Homburg/Saar, Germany), HD37 (anti-CD19) [35] and HP2/6 (anti-CD4) [36] to obtain CD8$^+$ cells or OKT8 (anti-CD8) [37] to isolate CD4$^+$ T cells. Negative isolation of T cells was performed with BioMag goat antimouse IgG Beads (Paesel + Lorei, Hanau, Germany). Purity of the population was determined by flowcytometry with CD4-FITC and CD8-FITC MoAb on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany). T cells were stimulated with α-CD3 (1 μg/ml) and α-CD28 MoAb (1 μg/ml, kindly provided by R.A. van Lier, Amsterdam, the Netherlands). To dissect unstimulated and stimulated T cells, cells were stained with CD45RA-FITC (Immunotech, Marseille, France) and CD45RO-PE (Immunotech).

Mixed lymphocyte reaction

HLA-A1$^-$ PBMC from healthy donors or HLA-A1-specific cytotoxic T cells (CTL) were stimulated with mitomycin C (Sigma, Germany) treated cells (100 μg mitomycin C/2×10^7 stimulator cells, 1 h, 37°C) at a ratio of 10 PBMC : 1 stimulator cell. Cells were pulsed with 1 μCi/well [methyl-^{3}H]thymidine (Amersham LIFE Science, UK) during the last 18 h of culture and harvested after 6 days on an Inotech harvester (Wallac, Freiburg, Germany) and radioactive thymidine incorporation was determined on a Microbeta Trilux Counter (Wallac).

Generation of alloreactive T cell lines

Ficoll-Hypaque separated PBMC from healthy HLA-A1$^-$ donors (1×10^6/ml) were incubated with mitomycin C-treated HLA-A1$^+$ .721 stimulator cells (1×10^5/ml) and human r-IL-2 (Biochrom KG) (30 U/ml). Viable cells were harvested weekly and restimulated with mitomycin C-treated .721 cells at a ratio of 10 : 1 in medium containing 30 U/ml of rIL-2. Cytotoxicity assays were carried out at day 6 after the last restimulation.

Cytotoxicity assay

Target cells, 2×10^6, were labelled with 200 μCi $Na^{51}CrO_4$ (Amersham-Buchler, Braunschweig, Germany) for 1 h. Increas-

ing numbers of effector cells were titrated to 5×10^3 target cells and incubated for 4 h at 37°C. Fifty μl of supernatant was assayed for ^{51}Cr-release in a Microbeta Trilux Counter (Wallac, Germany). Maximum release was determined by incubation of target cells in 100 μl 10% SDS and spontaneous release was determined by addition of medium. The percentage of specific release was calculated as percentage specific release = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. The whole assay was set up in triplicates.

Cytokine assays

The amount of cytokines (IL-2, IL-4, IL-10, IFN-γ) in mixed lymphocyte reaction (MLR) supernatants was determined by a sandwich enzyme immunoassay (R&D Systems, Wiesbaden, Germany). The minimal detectable dose of cytokines was found to be in the range of 10 pg/ml for each cytokine.

Western blot analysis

Cells were lysed for 15 min at 4°C in lysis buffer (TRIS/HCl 30 mM, pH 7·5, NaCl 150 mM, Triton X-100 1%, glycerol 10%, PMSF 1 mM, DTT 1 mM) followed by high-speed centrifugation. One hundred μg of lysate was separated on a 10–20% gradient SDS page and electroblotted onto Hybond ECL nitrocellulose membrane (Amersham Life Science, Braunschweig, Germany). Membranes were blocked for 1 h in PBS supplemented with 5% milk powder and 0·1% Tween 20. CD95L and β-actin were detected by mouse antibodies α-CD95L (Pharmingen, Clone G247-4) and α-β-actin (Sigma, clone AC-15) followed by subsequent incubation with goat anti-mouse IgG-HRP (Dianova, Germany) and detection by enhanced chemoluminescence (Amersham).

RESULTS

Induction of apoptosis by ISD

ISD are clinically used to prevent graft rejection and to suppress the cytotoxic action of autoaggressive T cells. Seven commonly used immunosuppressive drugs (DEX, MPA, CsA, FK506, RAP, MTX, CP) were tested for their ability to induce apoptosis in activated peripheral human T cells. To verify that T cells are dying by the process of apoptosis we compared three different methods to determine programmed cell death by ISD. Cell death of activated T cells after ISD treatment was determined by changes in cell size (FSC/SSC), by measurement of externalized phosphatidyserine on the membrane (Annexin V) or by analysing the DNA content of propidium-iodide stained nuclei (Nicoletti) (Fig. 1). We used the MoAb APO-1 that binds to the CD95 receptor as positive control for apoptosis induction. MTX and CP were the only ISD tested, which induced massive apoptosis in activated T cells. The different methods used are comparable and therefore apoptosis was determined in the following experiments by analysing FSC/SSC.

Apoptosis induction and modulation of CD95-induced apoptosis by ISD in Jurkat cells

As a model system for activated peripheral T cells we used the T cell leukaemic line Jurkat, which is a standard cell line to analyse mechanisms of apoptosis induction as AICD [13] or drug sensitivity [20]. In order to investigate whether ISD silence T cell effector functions or have direct apoptosis-inducing effects we determined specific apoptosis of Jurkat cells after drug treatment

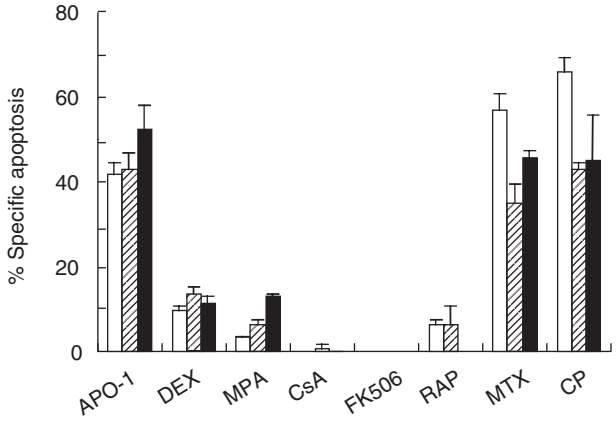

Fig. 1. Flow cytometric analysis of apoptosis induction in activated T cells by three different methods. Activated HLA-A1-restricted CTL were incubated with Apo-1 (1 μg/ml), DEX (10^{-7} M), MPA (100 ng/ml), CsA (100 ng/ml), FK506 (100 ng/ml), RAP (10 ng/ml), MTX (1000 ng/ml) and CP (1000 ng/ml) for 72 h. Percentage specific apoptosis was determined by measuring FSC/SSC, propidium iodide-stained DNA content (Nicoletti) and binding of annexin V-FITC to externalized phosphatidylserine. Data represent the mean of three different experiments. □, FSC/SSC; ▨, Nicoletti; ■, annexin V.

with increasing concentrations of MTX, CP, DEX, MPA, CsA, FK506 and RAP (Fig. 2a). While MTX and CP induced cell death over a wide range of concentrations, DEX, MPA, CsA, FK506 and RAP showed only minimal apoptosis-inducing effects even at high concentrations. Similar results were obtained with the T cell line H9 (data not shown). Induction of apoptosis by DEX in Jurkat and H9 cells only at high concentrations reflects the low level of glucocorticoid receptor expression by most T cell lines [38].

Treatment with cytotoxic drugs such as doxorubicin, cytarabine or MTX has been shown to sensitize apoptosis-resistant tumour cells for CD95-induced apoptosis [39]. We therefore analysed whether ISD also modulate CD95-mediated apoptosis. Jurkat cells were incubated with increasing amounts of the Apo-1 antibody in the presence or absence of ISD, and cell death was determined after 12 h (Fig. 2b). Drug concentrations were chosen according to concentrations, which may be achieved during clinical administration. While MTX and CP slightly increased CD95-mediated apoptosis, no effect of CsA, FK506, RAP, DEX and MPA was observed.

Modulation of AICD in Jurkat cells by ISD

AICD, mediated via the CD95/CD95L system, serves as a major mechanism to remove activated T cells from the periphery during the termination of an immune response. AICD can be induced in human T cell lines by TCR/CD3 triggering via immobilized MoAb OKT3. $CD3^+$ Jurkat cells were incubated in the presence or absence of OKT3 and the ISD CsA, FK506, RAP, MPA, DEX, MTX and CP. Induction of CD95L was assessed by Western blot analysis after 4 h (Fig. 3b) and apoptosis was determined by FACS analysis after 24 h (Fig. 3a). OKT3-induced up-regulation of CD95L and AICD was strongly inhibited in the presence of CsA and FK506. While DEX, MPA, MTX, RAP and CP slightly increased the level of CD95L expression, DEX, RAP and MPA did not alter OKT3-induced AICD. Treatment with MTX and CP, however, mediated increased AICD and also induced apoptosis

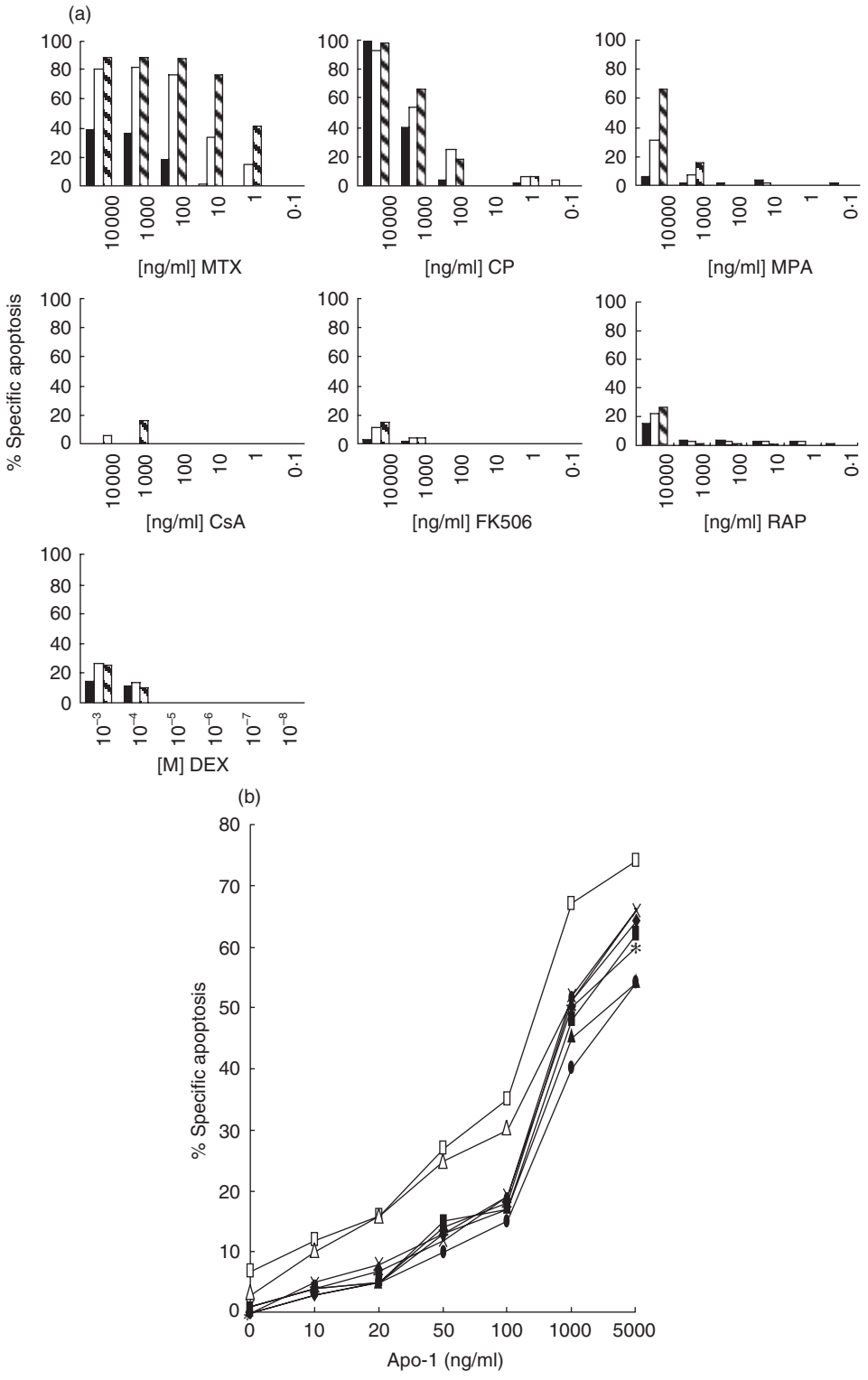

Fig. 2. MTX and CP induce cell death in Jurkat cells. Jurkat cells were incubated with increasing concentrations of MTX, CP, MPA, CsA, FK506, RAP and DEX and specific apoptosis was calculated after 24, 48 and 72 h by FACS analysis measuring FSC/SSC ■, 24 h; □, 48 h; ◪, 72 h. (a). Jurkat cells were incubated with increasing amounts of Apo-1 MoAb in the absence or presence of DEX, MPA, CsA, FK506, RAP, MTX and CP. (b) Viability was assessed by FACScan analysis after 12 h measuring FSC/SSC. ◆, Medium; ●, dexamethasone 10^{-7} M1; ▲, MPA 100 ng/ml; ×, CsA 100 ng/ml; *, FK506 100 ng/ml; ◆, RAP 10 ng/ml; △, MTX 1000 ng/ml; □, CP 1000 ng/ml. Results are representative of four distinct experiments showing similar effects.

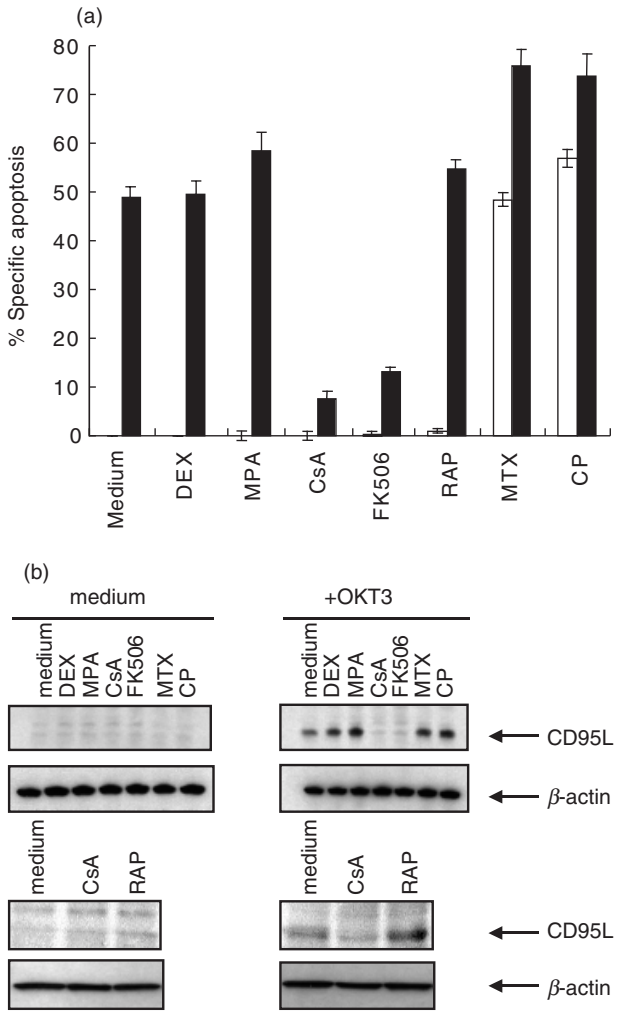

Fig. 3. Modulation of AICD of Jurkat cells by ISD. Jurkat cells were incubated with immobilized OKT3 (α-CD3, 100 μg/ml) or medium and ISD DEX (10^{-7} M), MPA (100 ng/ml), CsA (100 ng/ml), FK506 (100 ng/ml), RAP (10 ng/ml) MTX (1000 ng/ml) and CP (1000 ng/ml). (a) After 24 h specific apoptosis was determined by FACS analysis measuring FSC/SSC. □, Medium; ■, OKT3 (100 μg/ml). (b) After 4 h immunoblot analysis for CD95L and β-actin levels was performed; % specific apoptosis is the mean of one experiment of four performed and the gels shown are representative of four different experiments for both CD95L and β-actin.

in the absence of CD3-triggering. These data indicate that of all drugs tested, only CP and MTX slightly accelerate AICD of activated T cells.

MTX and CP induce apoptosis of activated T cells

Because leukaemic T cell lines such as Jurkat cells are derived from precursor T cell leukaemias and may reflect an activated state of immature T cells, we determined whether apoptosis induction by ISD is different in resting *versus* activated human $CD4^+$ and $CD8^+$ T cells. We either left the cells untreated or stimulated them via immobilized CD3 and CD28 MoAb and cultured them simultaneously in the presence of CsA, FK506, RAP, MPA, DEX, MTX and CP. After 4 days, proliferation (Fig. 4a) and cell death (Fig. 4b) was determined. All drugs except DEX, which did not inhibit proliferation of $CD8^+$ T cells after stimulation, reduced proliferation of $CD4^+$ and $CD8^+$ T cells. Similar to Jurkat T cells, MTX and CP directly induced apoptosis. Interestingly, CP treatment led to apoptosis of activated and resting T cells, while MTX mainly deleted stimulated T cells. DEX and RAP, however, triggered T cell death of resting T cells in the $CD8^+$ and $CD4^+$ population. These data indicate that MTX and CP inhibit T cell proliferation by direct apoptosis induction.

CP and MTX accelerate CD95-mediated apoptosis in human T cells

CD95-sensitivity of peripheral T cells develops during an immune response. After prolonged stimulation activated T cells turn into a CD95-sensitive state. To clarify whether ISD accelerate CD95-sensitivity, $CD4^+$ and $CD8^+$ T cell subsets were stimulated for 6 days by immobilized CD3 and CD28 MoAb and subsequently incubated with increasing amounts of APO-1 MoAb and ISD (Fig. 5). Cell death was determined in the $CD45RA^+$ (naive T cells) population and in the $CD45RO^+$ (activated T cells) population. As expected, only $CD45RO^+$ cells exhibited increased CD95-sensitivity, which was augmented further by co-incubation with CP and MTX. All other drugs tested did not exert an effect on CD95-mediated apoptosis. In T cells activated for 1 day and known to be constitutively CD95-resistant, a slightly additive effect in CD95-sensitivity was only found in the presence of CP, while other drugs tested had no effect on CD95-sensitivity (data not shown).

Modulation of AICD of peripheral T cells

AICD in peripheral T cells is mediated via the CD95/CD95L system in $CD4^+$ T cells [13] and by TNFR/TNF in most $CD8^+$ T cells [40]. To mimic AICD in human T cells *in vitro*, T cells were stimulated with anti-CD3 MoAb for 6 days and subsequently restimulated by PMA and ionomycin. In the presence or absence of ISD, CD95L expression was assessed after 7 h, and apoptosis-induction was measured after 18 h of restimulation. As illustrated in Fig. 6a, apoptosis of $CD4^+$ T cells decreased in the presence of CsA and FK506. Simultaneously, CD95L expression was down-regulated (Fig. 6b). DEX, MPA, MTX and CP did not modulate AICD of CD4 cells and did not change CD95L expression levels. Interestingly, $CD8^+$ T cells were not sensitive to induction of AICD independent of the presence of ISD (Fig. 6a). Western blot analysis indicated that CD95L was also up-regulated in the restimulated CD8 population but did not induce AICD. These results show that ISD do not accelerate autoregulatory apoptosis-induction of peripheral human T cells.

MPA, MTX and CP inhibit proliferation of alloantigen-activated PBMC

ISD may prevent T cell activation and proliferation in partially incomplete HLA-matched situations. It is unclear, however, whether they act on unprimed or activated T cells. Therefore we performed an HLA-A1 specific one-way mixed lymphocyte reaction (MLR). PBMC from HLA-A1 negative donors, representing naive, unprimed T cells, or HLA-A1-specific proliferating CTL, representing activated T cells, were incubated with the HLA-A1 expressing stimulator cells .721. ISD (DEX, MPA, CsA, FK506, RAP, MTX and CP) were added directly to the MLR and [^{3}H]TdR incorporation was measured after 6 days. All ISD, except DEX, inhibited the allo-specific proliferative response when added during the priming phase to naive T cells. When drugs were added to already preactivated, proliferating HLA-A1-

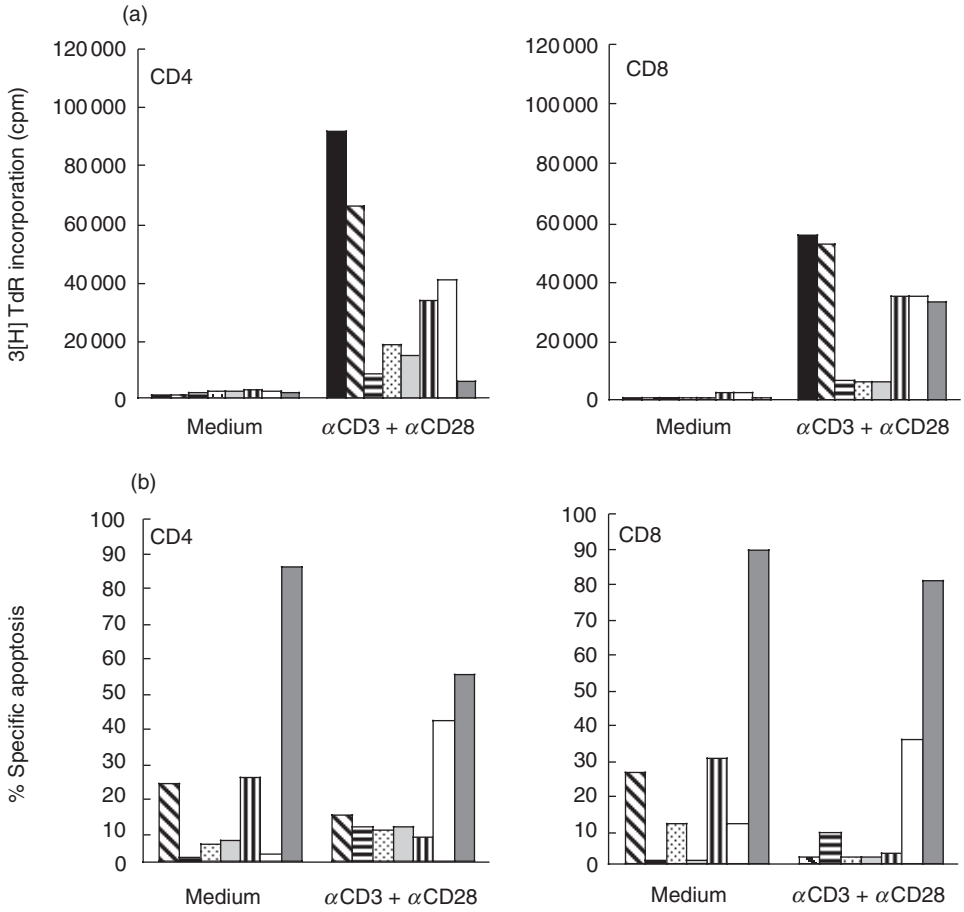

Fig. 4. Influence of ISD on T cell proliferation and apoptosis. Purified $CD4^+$ and $CD8^+$ human T cells were either stimulated with α-CD3 (1 μg/ml) and α-CD28 (1 μg/ml) MoAbs for 3 days or left untreated and were cultured together with DEX, MPA, CsA, FK506, RAP, MTX and CP. (a) At day 3 cells were labelled with ^{3}[H]thymidine and harvested after 18 h. (b) At day 4 specific apoptosis was determined by FACScan analysis measuring FSC/SSC. Experiments were performed three times and standard deviations were always <5% of the mean value. ■, Medium; ▨, dexamethasone 10^{-7} M; ▬, MPA 100 ng/ml; ⊡, CsA 100 ng/ml; ▩, FK506 100 ng/ml; ◧, RAP 10 ng/ml; □, MTX 1000 ng/ml; ▩, CP 1000 ng/ml.

specific CTL, only MTX and CP and to a lower extent MPA prevented proliferation (Fig. 7a). Similar results were obtained with the SH-EP neuroblastoma cell line as stimulator cells with the exception that also DEX inhibited proliferation of unprimed PBMC (data not shown). At day 2 of the MLR when naive HLA-A1$^-$ PBMC were used as effector cells, we determined the levels of Th1 (IL-2, IFN-γ) and Th2 cytokines (IL-4, IL-10) in the supernatant in the presence of CP, MTX and MPA. CsA was used as a control, known to down-regulate cytokine expression (Fig. 7b). While CsA reduced cytokine secretion, no reduction by MTX, CP and MPA was found. The release of the Th2-specific cytokine IL-4 could not be detected. These data indicate that all ISD tested suppress activation of naive T cells at the initiation step of an allo-response, but only MTX and CP and, to a lower extent, MPA reduce proliferation of antigen-induced T cell activation during the course of an immune response without affecting cytokine production. Considering the direct apoptosis-inducing capacities of MTX and CP on activated peripheral T cells, these results suggest that both drugs might induce deletion of antigen-specific T cells.

MTX and CP decrease cytotoxicity and induce apoptosis of alloantigen-specific cytotoxic T cells

Allograft rejection is mediated predominantly by activated cytotoxic T lymphocytes, which destroy target cells via the CD95/CD95L system or the perforin/granzyme pathway. Deletion of alloantigen-activated specific CTL strongly favours transplantation tolerance. Therefore we tested the cytotoxicity and apoptosis-inducing effects of ISD on an HLA-A1 specific human CTL line. This cell line was generated by several rounds of stimulation with HLA-A1 expressing .721 cells and cytotoxicity was assessed by a 4-h chromium release assay. The HLA-A1 specificity of the CTL line was tested against the non-HLA-A1 expressing parental cell lines. Cytotoxicity towards HLA-A1 expressing targets (C1R.A1, P1.A1/hβ_2m) was decreased in CTL only upon preincubation with MTX and CP (Fig. 8a). The effect of CP was consistently more prominent. All other ISD did not influence CTL activity significantly. In parallel, we examined whether drug treatment induced apoptosis of antigen-specific CTL. As in the previous experiments with activated T cells, only MTX and CP induced cell death in 50% of the CTL population,

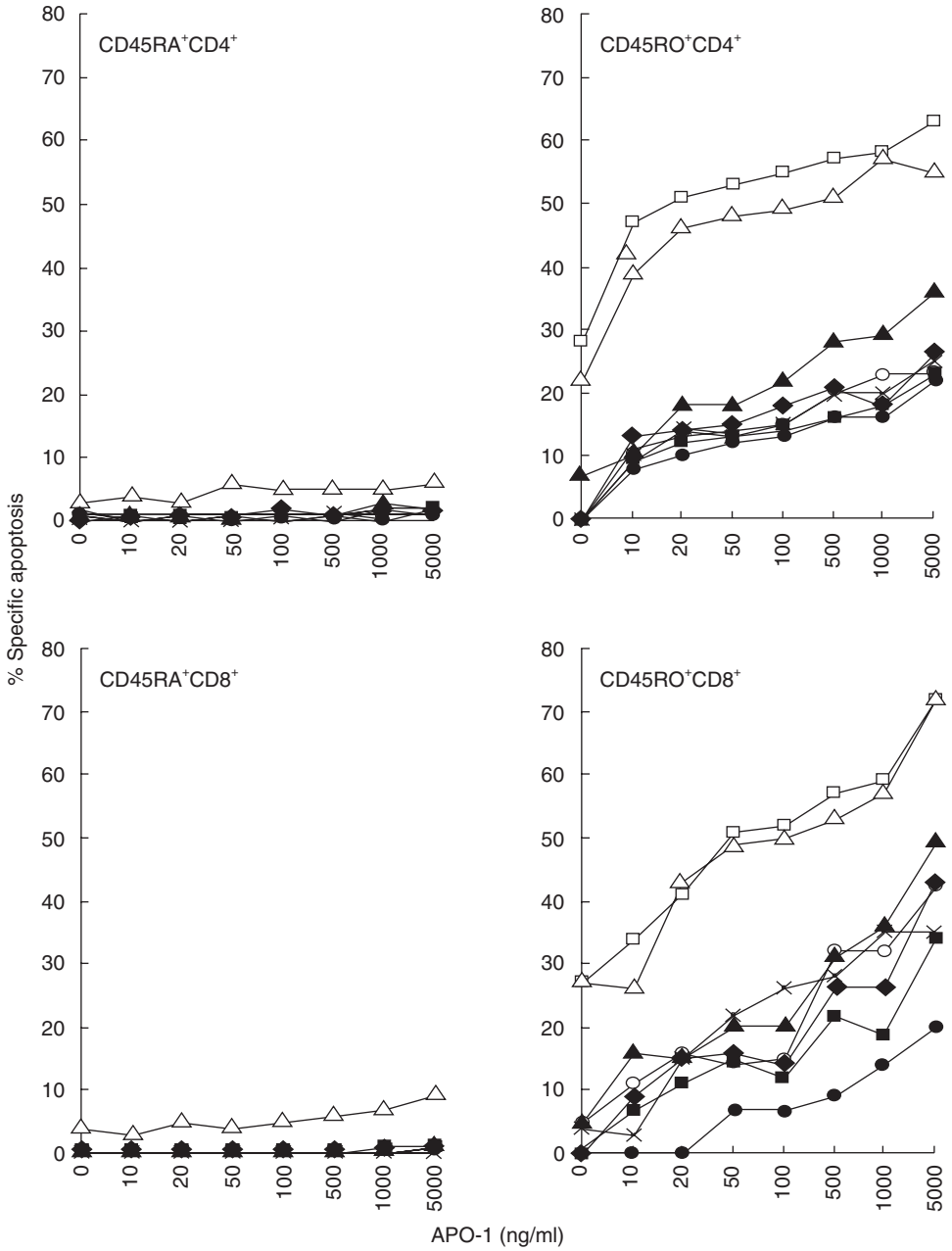

Fig. 5. MTX and CP accelerate CD95-mediated apoptosis of stimulated T cells. $CD4^+$ and $CD8^+$ human T cells were stimulated with α-CD3 (1 μg/l) and α-CD28 (1 μg/ml) MoAbs for 6 days and afterwards incubated with increasing concentrations of APO-1 in the absence or presence of DEX (10^{-7} M), MPA (100 ng/ml), CsA (100 ng/ml), FK506 (100 ng/ml), RAP (10 ng/ml), MTX (1000 ng/ml) and CP (1000 ng/ml). After 24 h cells were double-stained with MoAb CD45RA-FITC and CD45RO-PE and specific apoptosis was determined by FACScan analysis measuring FSC/SSC. Values are the mean of triplicates and represent one of four different experiments. ◆, Medium; ■, dexamethasone; ▲, MPA; ○, CsA; ×, FK506; ●, RAP; □, MTX; △, CP.

indicating that these drugs may directly delete activated antigen-specific CTL (Fig. 8b). Furthermore, these results also show that appropriate antigen stimulation does not counteract the apoptosis-inducing effect of CP and MTX.

DISCUSSION

Clinical results of bone marrow transplantation and organ transplantation have been improved significantly in the past 2 decades due to better insights into the immunobiology of graft rejection and GVHD and the development of new ISD. While ISD reduce the risk of GVHD and allograft rejection they also increase the incidence of infections and malignancies due to a global suppression of the endogenous immune system [1]. Alloreactive T cells may be inhibited at several levels, such as activation of naive T cells, prevention of transformation into effector cells or silencing of CTL effector function. Ideally, alloreactive T cells should be deleted to induce stable transplantation tolerance. Also in

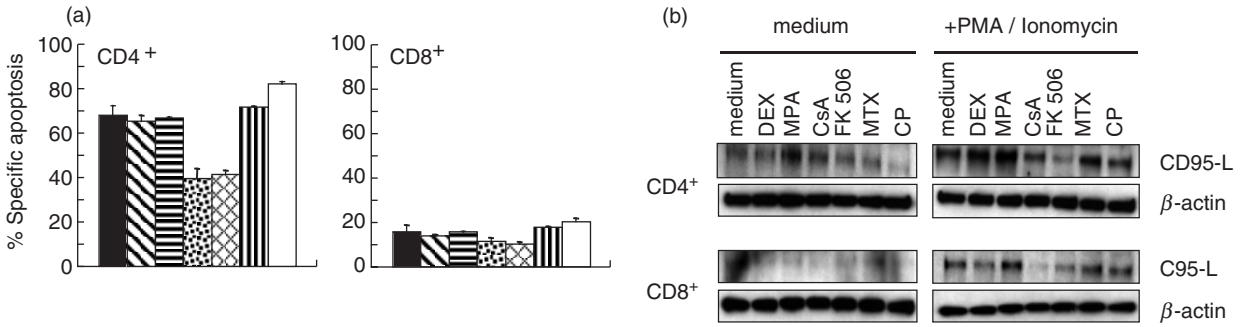

Fig. 6. ISD do not accelerate AICD in human peripheral T cells. Purified human $CD4^+$ and $CD8^+$ T cells were activated for 6 days with OKT3 (100 ng/ml) and then restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 16 h (a) or 7 h (b) in the presence or absence of DEX (10^{-7} M), MPA (100 ng/ml), CsA (100 ng/ml), FK506 (100 ng/ml), MTX (1000 ng/ml) or CP (1000 ng/ml). (a) Viability was assessed by FACScan analysis measuring FSC/SSC. ■, Medium; ▧, dexamethasone; ▤, MPA; ▩, CsA; ▨, FK506; ▮, MTX; □, CP. (b) Immunoblot analysis for CD95L and β-actin (protein loading control) levels; % specific apoptosis is the mean of one experiment of three performed and the gels shown are representative of three different experiments for both CD95L and β-actin.

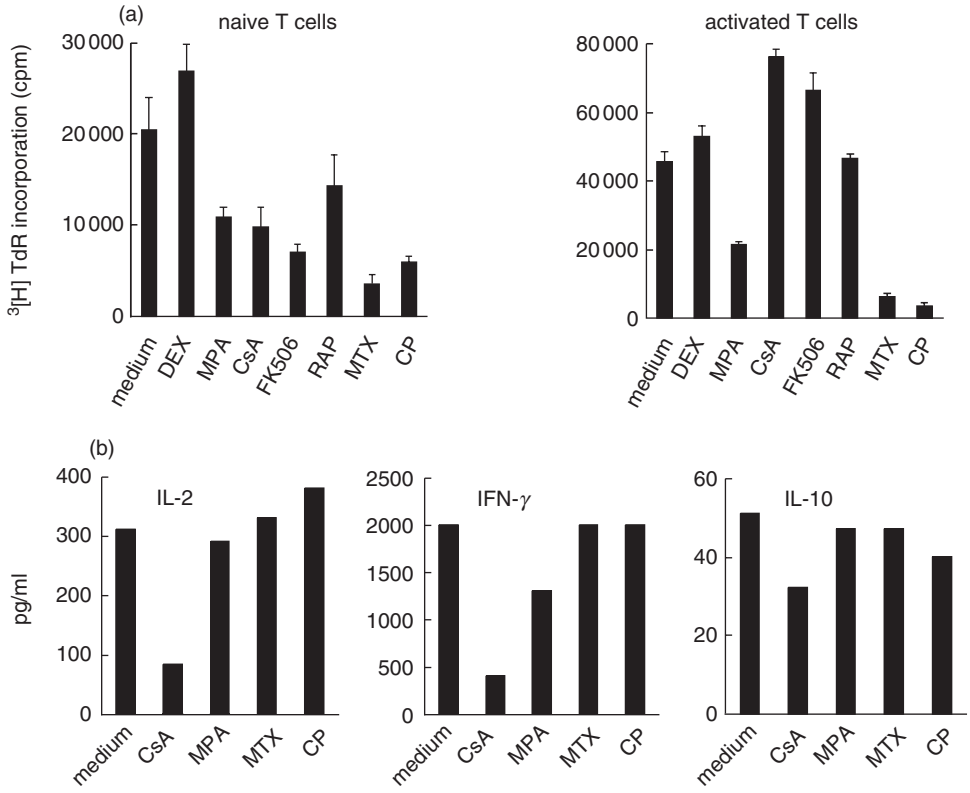

Fig. 7. MPA, MTX and CP inhibit proliferation of preactivated antigen-specific T cells. PBMC from healthy $HLA\text{-}A1^-$ donors (naive T cells) or HLA-A1 specific CTL (activated T cells) were stimulated with $HLA\text{-}A1^+$ .721 cells in the absence or presence of DEX (10^{-7} M), MPA (100 ng/ml), CsA (100 ng/ml), FK506 (100 ng/ml), RAP (10 ng/ml), MTX (1000 ng/ml) or CP (1000 ng/ml). At day 5 cells were labelled with ^{3}[H]thymidine and harvested after 18 h (a). Cytokine production of .721 stimulated $HLA\text{-}A1^-$ PBMC was determined at day 2 after antigen stimulation (b). Data are representative of three experiments.

autoimmune diseases mediated by autoreactive T cells deletion of antigen-specific T cells is the major goal of an effective therapy. Although most ISD are in clinical use for many years, the effect of these drugs in the different phases of T cell activation and effector function during immune responses is understood only partially. Therefore we investigated whether ISD therapy inhibits T cell activation and proliferation in human leukaemic T cell lines and peripheral T cells leading to T cell silencing, or induces apoptosis causing deletion of T cells.

In the present study we found, among the ISD tested, only CP and MTX induced apoptosis in the Jurkat T cell line, peripheral human resting and activated T cells and allospecific CTL lines. DEX, MPA, CsA, RAP and FK506 did not induce cell death or modulate CD95-triggered apoptosis-mechanisms but inhibited

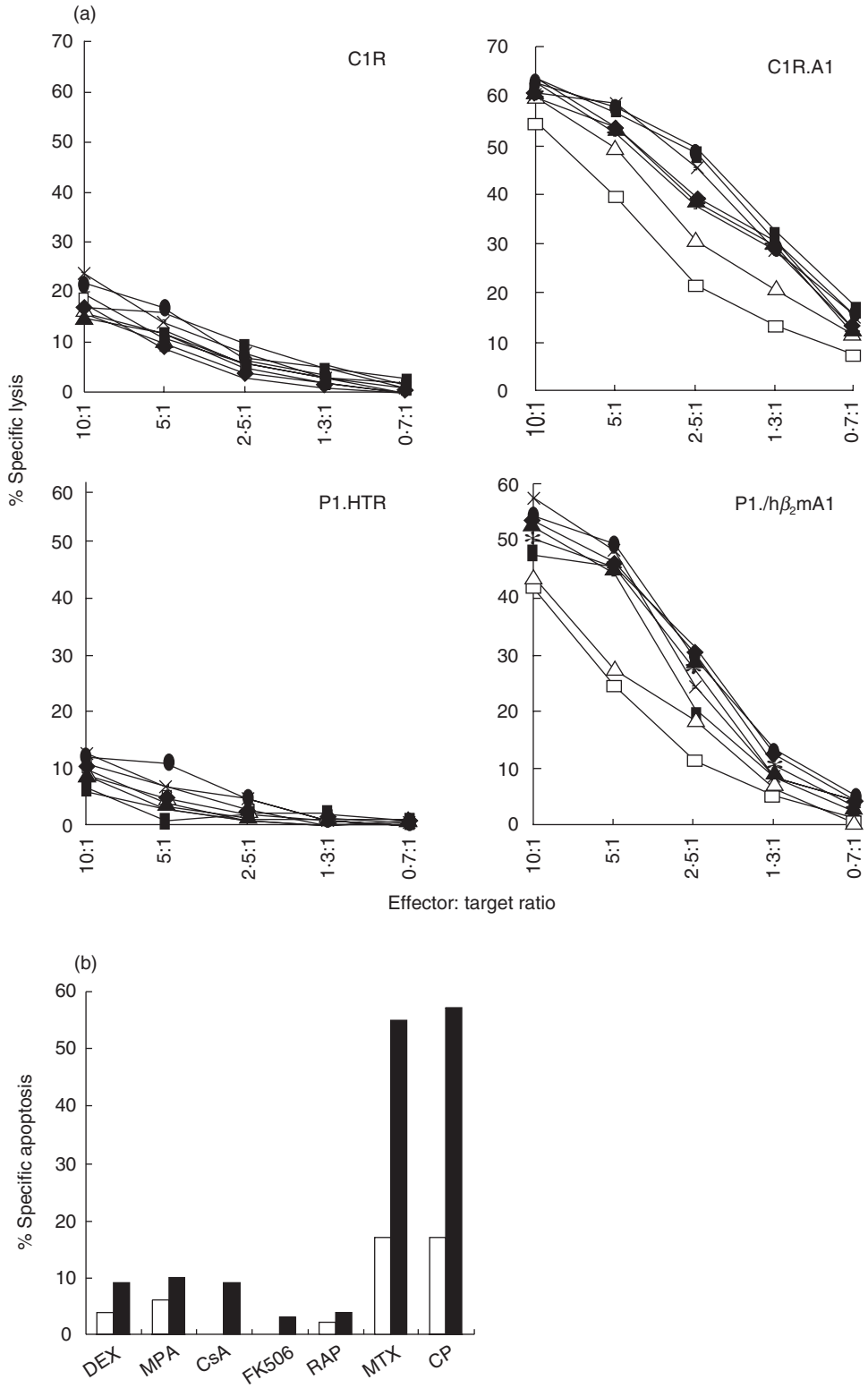

Fig. 8. MTX and CP decrease cytotoxicity and induce apoptosis of allo-antigen specific CTL. HLA-A1 specific CTL were incubated at day 5 after the last restimulation with in DEX (10^{-7} M), MPA (100 ng/ml), CsA (100 ng/ml), FK506 (100 ng/ml), RAP (10 ng/ml), MTX (1000 ng/ml), CP (1000 ng/ml) or medium. After 24 h a standard chromium release assay was performed. The HLA-A1-positive cell lines C1R.A1 and P1.A1/hβ_2m and the untransfected cell lines C1R and P1.HTR were used as ^{51}Cr-labelled targets (a). ◆, Medium; ■, dexamethasone; ▲, MPA; ×, CsA; *, FK506; ●, RAP; △, MTX; □, CP. At 24 and 48 h after ISD incubation specific apoptosis was determined by FACScan analysis measuring FSC/SSC (b). □, 24 h; ■, 48 h. One representative experiment out of six performed is shown and data represent the mean value of triplicates.

proliferation of naive T cells, suggesting that these drugs do not mediate elimination of T cells. Although DEX is known to induce massive cell death in immature thymocytes [17] the lack of DEX-induced apoptosis in activated T cells at concentrations which may be comparable to concentrations achieved during clinical administration has been described [41]. Furthermore, interleukins such as IL-2, IL10 and IL-4 which are produced by activated T cells counteract the action of DEX-induced apoptosis [42], explaining why naive T cells are more susceptible to apoptosis induction by DEX than proliferating activated T cells.

Only CP and MTX induce direct apoptosis in Jurkat T cells and peripheral T cells while DEX, MPA, CsA, RAP and FK506 showed no effect. The ability of CP to delete activated T cells has already been suggested in mouse models. Mice which were primed with minor antigen incompatible spleen cells and simultaneously treated with CP showed clonal deletion of activated allogeneic T cells in the periphery and the thymus [43,44]. Interestingly, we found that CP induced apoptosis in activated as well as in resting T cell populations, while MTX only mediated apoptosis of activated T cells, as described previously [22].

Induction of apoptosis in activated T cells might be induced by an accelerated CD95-sensitivity, e.g. through chemotherapeutic drugs such as doxorubicine, cytarabine, MTX and 6-mercaptopurine [39] or by an increase in autocrine T cell suicide (AICD) [13]. We observed a minimal increase of CD95-sensitivity induced by MTX and CP in Jurkat cells. CP and MTX had a stronger specific effect on activated peripheral $CD4^+$ and $CD8^+$ T cells. All other ISD tested did not modulate CD95-sensitivity in Jurkat cells or in peripheral T cells. AICD in Jurkat T cells [45] and peripheral human T cells [46] is mediated mainly by an increased production and release of CD95L. Therefore we hypothesized that ISD might induce an accelerated AICD by increased CD95L production. Of all drugs tested, however, only MTX and CP slightly increased AICD of Jurkat cells or peripheral T cells. On the contrary, CsA and FK506 even inhibited AICD in Jurkat T cells or in activated peripheral human $CD4^+$ T cells by down-regulation of CD95L. DEX, MPA and RAP did not increase AICD in Jurkat T cells or human peripheral T cells, suggesting that immunosuppressive therapy does not enhance autoregulatory apoptosis. Recently for TRAIL (Apo2L), a role has also been described in the induction of AICD in Jurkat T cells [47]. As more than 90% of cell death in CD3-triggered Jurkat cells in our system can be blocked with $F(ab')_2$ APO-1 fragments, by inhibition of the interaction of CD95 with its ligand, the CD95 system is the major mechanism of AICD in these cells (data not shown).

ISD are used to inhibit T cell effector function in partially incomplete HLA-matched organ transplantations. We therefore analysed the effect of ISD on HLA-specific T cell proliferation and T cell cytotoxicity. MTX and CP and to lower extent MPA prevented proliferation of activated T cells suggesting that these drugs are able to inhibit the expansion of primed and activated antigen-specific T cells after organ transplantation and in the treatment of autoimmune diseases. All other ISD, however, did not affect activated T cells, but only inhibited T cell proliferation when administered at the time of first antigen contact. Considering the direct apoptosis-inducing capacities of MTX and CP we assume that inhibition of proliferation of activated T cells is mediated via apoptosis because both drugs did not affect T cell cytokine secretion. To clarify whether MTX and CP also suppress cytotoxicity and delete activated cytotoxic T cells, we established human HLA-A1 specific CTL lines. Again, decreased cytotoxicity towards HLA-A1 positive target cells and induction of apoptosis was observed only in the presence of MTX and CP. RAP, which has been described to inhibit mouse CTL induction when added at the beginning of MHC-unrestricted stimulation [48], also could not block CTL cytotoxicity or induce apoptosis when added to preactivated HLA-A1 restricted CTL.

The importance of apoptosis induction for peripheral transplantation tolerance has been demonstrated in recent reports [26,49]. Wells *et al*. showed that mice transgenic for bcl-x_L expression in T cells or IL-2 deficient mice are resistant to the induction of transplantation tolerance due to defective passive or active T cell apoptosis pathways. The blockade of costimulation by inhibition of CD28–B7 and CD40–CD40L interaction in combination with immunosuppressive CsA treatment also did not lead to allograft tolerance [49]. These findings support the concept that direct depletion of alloreactive T cells is needed during the inductive phase of tolerance to reduce the number of the alloreactive T cell pool. In the following maintenance phase anergy or regulatory mechanisms may control the level of autoaggressive cells. Clinical observations with allotransplanted patients support these findings. For example, a combination of MTX, CsA and prednisone was found to be more effective in preventing acute GVHD than the combination of CsA and prednisone alone [50–52], supporting the idea that MTX deletes activated T cells that have escaped from proliferation blockade by other ISD. Recent data from mice with lupus nephritis also indicate that a treatment, which combines the inhibition of T cell activation by CTLA4Ig and the induction of apoptosis in immune cells by CP, reduced renal disease and prolongs survival [53]. The use of CP in liver and kidney transplantations in the mouse system also indicated that CP induces transplantation tolerance by depletion of immunocompetent cells [54,55].

In conclusion, our data show that of all drugs tested only CP and MTX have apoptosis-inducing capacities on activated T cells. Drugs such as CsA, FK506, RAP, DEX and MPA inhibit proliferation and expansion after antigen priming, indicating that T cells are silenced and not deleted. This finding may be useful for a clinical design of immunosuppressive regimens which aim to delete antigen-specific T cells by a combination of antiproliferative and apoptosis-inducing effects in order to reduce the amount of alloreactive T cells after organ transplantation or to remove autoreactive T cells in the treatment of autoimmune diseases.

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