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Contrasting Impacts of Immunosuppressive Agents (Rapamycin, FK506, Cyclosporin A, and Dexamethasone) on Bidirectional Dendritic Cell-T Cell Interaction During Antigen Presentation[1]

Hiroyuki Matsue,[2] Chendong Yang, Keiko Matsue, Dale Edelbaum, Mark Mummert, and Akira Takashima

Rapamycin (RAP), tacrolimus (FK506), cyclosporin A, and glucocorticoids represent modern and classic immunosuppressive agents being used clinically. Although these agents have distinct molecular mechanisms of action and exhibit different immunoregulatory profiles, their direct influences on Ag presentation processes remain relatively unknown. Here we report quantitative and qualitative differences among the above four immunosuppressants in their impact on Ag-specific, bidirectional interaction between dendritic cells (DC) and $CD4^+$ T cells. In the presence of relevant Ag, bone marrow-derived DC delivered activation signals to $CD4^+$ T cells isolated from the DO11.10 TCR transgenic mice, leading to clonal expansion; secretion of IFN-γ, IL-2, and IL-4; and surface expression of CD69. Conversely, DO11.10 T cells delivered maturation signals to DC, leading to IL-6 and IL-12 production and CD40 up-regulation. FK506 (10^{-10}–10^{-8} M) and cyclosporin A (10^{-9}–10^{-7} M) each blocked efficiently and uniformly all the changes resulting from intercellular signaling in both DC→T cell and T cell→DC directions. Dexamethasone (10^{-9}–10^{-6} M) suppressed all changes, except for CD69 up-regulation, rather incompletely. Remarkably, RAP (10^{-10}–10^{-8} M) efficiently inhibited DC-induced T cell proliferation and T cell-mediated CD40 up-regulation by DC without abrogating other changes. Interestingly, T cell-independent DC maturation triggered by LPS stimulation was inhibited by dexamethasone, but not by other agents. Our results demonstrate contrasting pharmacological effects of RAP vs calcineurin inhibitors on Ag presentation, thus forming a conceptual framework for rationale-based selection (and combination) of immunosuppressive agents for clinical application. *The Journal of Immunology*, **2002, 169: 3555–3564.**

Immune dysregulation plays pathogenic roles in a wide spectrum of inflammatory diseases, including hypersensitivity responses to environmental Ag (allergic disorders), false recognition of self-Ag (autoimmune diseases), and robust immune attack against allo-Ag (graft rejection and graft-vs-host disease (GVHD).[3] Rapamycin (RAP; or sirolimus), tacrolimus (FK506), cyclosporin A (CyA), and glucocorticoids represent relatively new and classic therapeutics currently used for these disorders.

CyA and FK506 bind to cyclophilin A and 12-kDa FK506 binding protein (FKBP12), respectively. The CyA-cyclophilin A and the FK506-FKBP12 complexes bind to the same target, calcineurin, thereby inhibiting TCR-mediated signal transduction pathways (e.g., NFAT-dependent transcription of cytokine genes) (1–3). Although RAP and FK506 share the same receptor FKBP12, the RAP-FKBP12 complex binds to a distinct molecular target, known as mammalian target of RAP. The RAP-FKBP12-mammalian target of RAP complex inhibits phosphorylation of p70 S6 kinase and phosphorylated heat- and acid-stable protein-1, thereby inhibiting protein synthesis at the translational level. The same complex also blocks cytokine-mediated signal transduction pathways (via inhibiting the dissociation of $p27^{kip1}$ from cyclin C kinases) and CD28-mediated NF-κB activation pathways (via inhibiting IκBα phosphorylation) (3–6). Glucocorticoids inhibit gene transcription directly by competing for DNA binding sites in the promoter regions or indirectly by cross-coupling with many transcription factors, including AP-1 and NF-κB (7–9). A synthetic glucocorticoid, dexamethasone (DEX), has been reported recently to attenuate early steps of TCR signaling by affecting the membrane compartmentalization of key transducing molecules (10). Thus, the four immunosuppressants differ from each other in their molecular mechanisms of action.

T cells act as major effectors, causing tissue damage in immunological diseases, and thus may serve as a relevant cellular target for immunosuppressive agents. In fact, many studies in early 1990s documented potent activities of CyA, FK506, and DEX to inhibit production of IL-2 and other cytokines, IL-2R expression, and proliferation by T cells in response to mitogenic stimuli, such as lectins, phorbol ester plus calcium ionophore, or anti-CD3 mAb plus anti-CD28 mAb (11–16). By contrast, RAP inhibits mitogen-induced proliferation of T cells without affecting cytokine production or cytokine receptor expression, and it also suppresses T cell proliferative responsiveness to exogenously added growth factors (12, 13). Thus, unlike calcineurin inhibitors, which primarily block TCR-dependent signaling pathways (signal 1), RAP appears to

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[3] Abbreviations used in this paper: GVHD, graft-vs-host disease; CD40L, CD40 ligand; CyA, cyclosporin A; DC, dendritic cells; DEX, dexamethasone; FK506, tacrolimus; FKBP12, 12-kDa FK506 binding protein; PI, propidium iodine; RAP, rapamycin.

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inhibit costimulatory signaling pathways (signal 2) and cytokine receptor-mediated signaling pathways (signal 3) (17). Dendritic cells (DC), which are responsible for initiating Ag-specific T cell responses (18), are a second putative cellular target. FK506, CyA, and DEX have been reported to inhibit the generation (or differentiation) of DC from their progenitors in vitro and/or their subsequent maturation induced by CD40 ligand (CD40L), TNF-α, or LPS (19–23). RAP has been shown recently to trigger apoptosis of DC progenitors in vitro without affecting their CD40L-induced maturation (24). Thus, each immunosuppressant can alter some functions of T cells and DC.

During Ag presentation, DC deliver signals 1–3 to naive T cells, leading to their priming, differentiation, and clonal expansion. At the same time, DC appear to receive maturation signals from responding T cells. For example, coupling of CD40 (on DC) with CD40L (on T cells) triggers secretion of IL-12 and other cytokines by DC and augments their T cell stimulatory capacity (25–28). We reported previously that an immature DC line, XS52, exhibited a series of maturational changes upon Ag-specific interaction with T cell clones (29–31). A key question would then concern potential impacts of immunosuppressants on such bidirectional DC-T cell communication during Ag presentation. Here we address this question using an in vitro Ag presentation system in which bone marrow-derived DC and $CD4^+$ T cells isolated from DO11.10 TCR transgenic mice are cocultured in the presence of relevant Ag.

Materials and Methods

Animals

BALB/c mice (6- to 8-wk-old females) and DO11.10 transgenic mice (32) (6- to 10-wk-old animals) were used in this study. DO11.10 mice were housed under specific pathogen-free conditions in the Animal Research Center facilities at University of Texas Southwestern Medical Center.

Reagents and Abs

DEX, CyA, and RAP were purchased from Sigma (St. Louis, MO) and dissolved in ethanol to prepare stock solutions at 10^{-2}, 10^{-3}, and 10^{-4} M, respectively. FK506 obtained from Calbiochem (La Jolla, CA) was dissolved in ethanol at a stock concentration of 10^{-4} M. These reagents were added to the culture medium at various concentrations with the constant ethanol concentration of 0.1%. The $OVA_{323–339}$ peptide was synthesized at the Biopolymers Facility, University of Texas Southwestern Medical Center. None of the reagents contained detectable amounts of endotoxin as tested by the OCL-100 system (BioWhittaker, Walkersville, MD). LPS (*Escherichia coli* 026:B6) and Con A were obtained from Sigma and Amersham Pharmacia Biotech (Piscataway, NJ), respectively. The mAb KJ1.26, which recognizes the transgenic TCR complex expressed by DO11.10 T cells, and its isotype-matched control IgG were purchased from Caltag (Burlingame, CA). Other mAbs were purchased from BD PharMingen (San Diego, CA). Recombinant mouse GM-CSF, IL-4, and IL-12 were purchased from R&D Systems (Minneapolis, MN).

Cell preparations

$CD4^+$ T cells were purified from spleens of DO11.10 mice using magnetic beads (Dynabeads Mouse CD4-L3T4 and DETACHaBEAD Mouse CD4, Dynal, Oslo, Norway), followed by depletion of $I\text{-}A^+$ contaminants by anti-MHC class II (I-A) microbeads (Miltenyi Biotec, Auburn, CA). The resulting T cell preparations containing 97–99% $CD4^+$ cells were used without further purification. Short term (7-day) Th1 or Th2 cultures were generated from DO11.10 $CD4^+$ T cells in the presence of IL-12 plus anti-IL-4 mAb or IL-4 plus anti-IL-12 mAb, respectively (33). Bone marrow-derived DC were generated from BALB/c mice in complete RPMI 1640 medium (34) in the presence of 10 ng/ml GM-CSF as described previously (35, 36). The resulting DC preparations harvested on days 5–7 contained 72–92% $CD11c^+$ cells and expressed a characteristic phenotype of immature DC. In some experiments $CD11c^+$ fractions (>99% purity) were isolated from the above bone marrow-derived DC preparations using MACS CD11c microbeads (Miltenyi Biotec).

In vitro Ag presentation assays

Freshly isolated DO11.10 T cells (2.5×10^5 cells/ml) were cocultured with gamma-irradiated (1500 rad) bone marrow-derived DC (5×10^4 cells/ml) and 2 μg/ml OVA peptide. To test the secondary activation, Th1 and Th2 T cells (5×10^4 cells/ml) were cocultured with gamma-irradiated DC (5×10^3 cells/ml). Cells were pulsed for 8 h with [^{3}H]thymidine (1μCi/well) and were harvested on day 3 for the primary response or on day 2 for the secondary response (34).

Phenotype, cell viability, and apoptosis assays

DO11.10 T cells and bone marrow-derived DC were cocultured for 20 or 24 h with OVA peptide in the presence of each immunosuppressant. The samples were then examined for CD69 expression within the $KJ1.26^+$ T cell populations (expressing the transgenic TCR complex) and for the expression of CD40, $I\text{-}A^d$, CD80, and CD86 within the $CD11c^+$ DC populations using FACSCalibur (BD Immunocytometry Systems, San Jose, CA). Viabilities of T cells and DC were assessed by measuring propidium iodine (PI) uptake by the $KJ1.26^+$ populations and $CD11c^+$ populations, respectively. To examine an early apoptotic change, we counted the numbers of PI-negative/annexin V-positive cells after staining with Cy5-conjugated annexin V (BD PharMingen).

Cytokine analyses

To study cytokine release by DC, supernatants from cocultures of DC (4×10^5 cells/ml) and T cells (2×10^6 cells/ml) were examined by ELISA for IL-6, IL-12 p40, and IL-12 p70. To study cytokine release by T cells, supernatants from cocultures of T cells (1×10^5 cells/ml) and DC (1×10^4 cells/ml) were examined for IL-2, IL-4, and IFN-γ. To assess intracellular cytokine deposition, DC (2×10^6 cells/ml) and T cells (8×10^6 cells/ml) were cocultured for 22 h in the presence of brefeldin A added during the last 5-h period (to inhibit intracellular transport processes). Subsequently, samples were labeled with FITC-anti-CD11c mAb or FITC-anti-CD4 mAb, fixed and permeabilized with the Cytofix/Cytoperm solution (BD PharMingen), and stained with PE-conjugated mAb against IL-2, IL-4, IFN-γ, IL-6, or IL-12.

CCR7 mRNA analysis

$CD11c^+$ DC fractions sorted by magnetic beads were examined for CCR7 mRNA expression by real-time RT-PCR (37). Briefly, total RNA (1 μg/sample) was reverse transcribed using Omniscript reverse transcriptase (Qiagen, Valencia, CA), and the resulting cDNA (16 ng/sample) was subjected to real-time, semiquantitative PCR using the iCycler (Bio-Rad, Hercules, CA) with SYBR Green I (Qiagen). Based on the threshold cycle numbers for CCR7 and β-actin, the relative expression levels for CCR7 were calculated using the iCycler software. The following primers were used to amplify CCR7 signals: CAAGAAGGATGTGCGGAACT (5′ primer) and TAGGCCCAGAAGGGAAGAAT (3′ primer).

Statistical analyses

Dose-dependency data were analyzed using the nonlinear regression equation library in the SigmaPlot 2001 software (SPSS, Chicago, IL).

Results

Development of an in vitro Ag presentation system

An overwhelming majority of the $CD4^+$ T cells isolated from DO11.10 TCR transgenic mice expressed a characteristic phenotype of naive T cells, i.e., $CD25^-$ (>95%), $CD69^-$ (>97%), $CD62L^{high}$ (>84%), $CD44^{low}$ (>91%), and $CD45RB^{high}$ (>98%; data not shown). These populations were used without further purification as responder T cells. Bone marrow-derived DC cultures generated from BALB/c mice were used as relevant APC; these cultures propagated in the presence of GM-CSF alone for 5–7 days contained 72–92% $CD11c^+$ cells. As reported previously (19, 38), these $CD11c^+$ populations expressed relatively low amounts of I-A molecule and costimulatory molecules (CD40, CD80, and CD86; Fig. 1*A*). To further assess the state of maturation of these DC preparations, we examined mRNA expression for CCR7, which is known to be up-regulated upon DC maturation (39–41). Real-time PCR analyses revealed that CCR7 mRNA expression was detectable in a magnetic bead-purified $CD11c^+$ DC population (Fig. 1*B*). Importantly, the relative expression level of CCR7

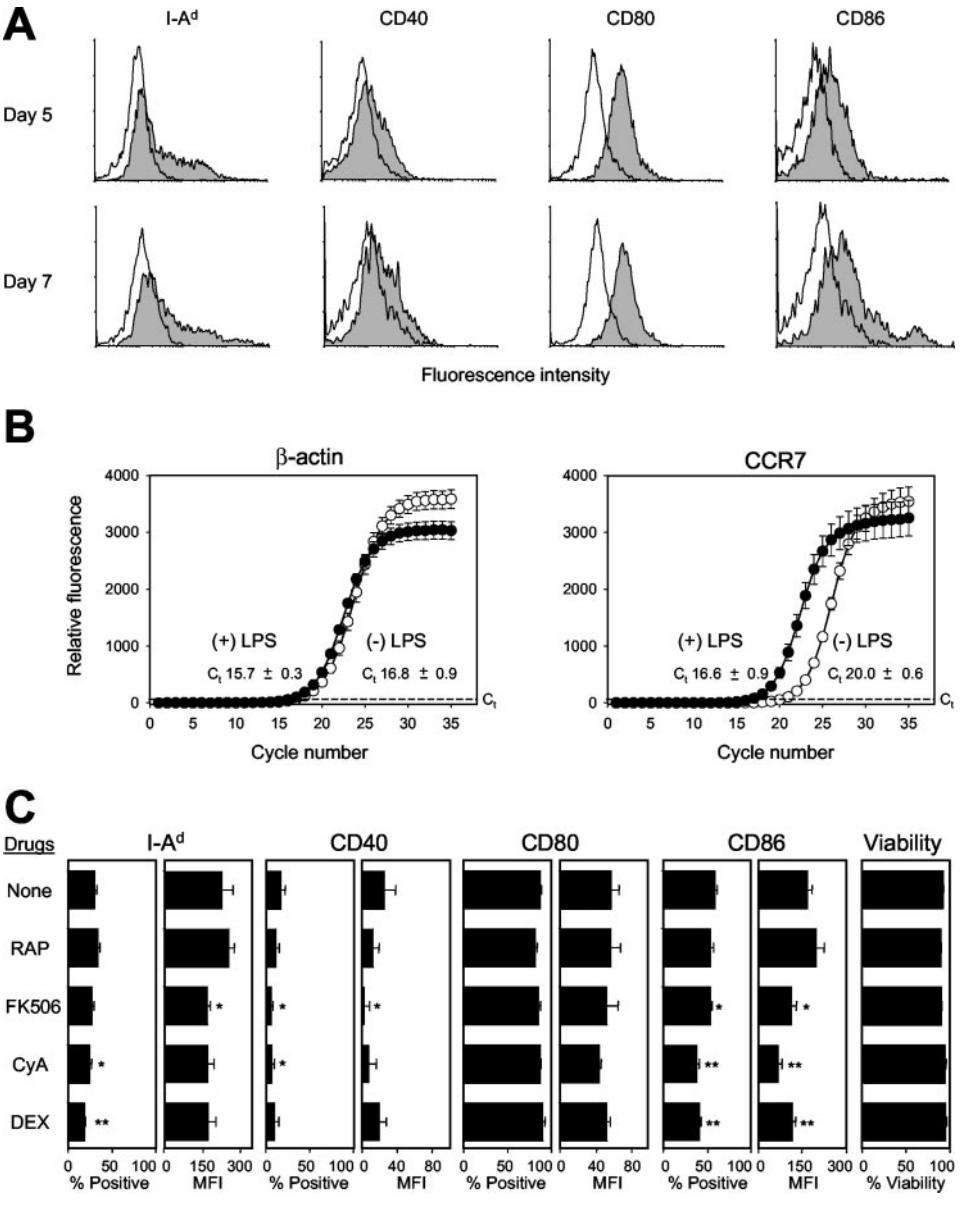

FIGURE 1. Direct effects of immunosuppressive agents on bone marrow-derived DC. *A*, Bone marrow-derived DC harvested on day 5 (*top*) and day 7 (*bottom*) were stained with mAbs for I-Ad, CD40, CD80, and CD86 (filled histograms) or isotype-matched control IgG (open histograms). *B*, The CD11c$^+$ fractions purified from bone marrow-derived DC cultures (day 5) were incubated for 16 h in the presence (●) or the absence of 100 ng/ml LPS (○). RNAs isolated from these cells were then subjected to real-time RT-PCR analyses for β-actin (*left*) and CCR7 (*right*). The curves represent relative fluorescence intensities of the resulting PCR products after the indicated cycles of amplification (mean ± SEM) from nine independent PCR reactions for each condition. Based on the cycle threshold (C_t) values, i.e., the numbers of PCR cycles required for detection of the relevant PCR products, it was calculated that the CCR7 mRNA expression levels normalized by β-actin mRNA expression in nonstimulated DC were 24.7 ± 18.2% of those in LPS-stimulated DC. *C*, Bone marrow-derived DC (day 5) were cultured for 2 additional days in the presence or the absence of RAP (10^{-8} M), FK506 (10^{-8} M), CyA (10^{-6} M), or DEX (10^{-6} M) and then examined for surface expression of the indicated molecules. The same samples were examined for cell viability by PI uptake. Data shown are the mean ± SD ($n = 3$) percentage of positive cells, mean fluorescence intensity (MFI), and percentage of viable cells within the CD11c$^+$ populations. Statistically significant differences compared with the nontreated samples are indicated with asterisks (*, $p < 0.05$; **, $p < 0.01$). All data shown in this figure are representative of at least two independent experiments.

mRNA in this population was rather modest (~20%) compared with that observed after LPS stimulation. These results indicate the relative immaturity of the DC preparations used in the present study.

As reported previously (42, 43), a short term (48-h) exposure of the above DC preparations to FK506 (10^{-8} M) or CyA (10^{-6} M) caused a relatively modest reduction in surface expression of I-A, CD40, and CD86 (Fig. 1*C*). DEX (10^{-6} M) also down-regulated I-A and CD86 expression marginally, whereas RAP (10^{-8} M) showed no significant inhibition. DC viability, as assessed by PI uptake by the CD11c$^+$ populations, remained unchanged after exposure to any drugs. These observations suggest that the four immunosuppressants, when tested in the absence of other stimuli, only modestly alter the surface phenotype of our DC preparations.

Bone marrow-derived DC (generated from BALB/c mice) induced robust proliferation of CD4$^+$ T cells purified from the DO11.10 TCR transgenic mice (on a BALB/c background) in the presence of OVA peptide in a manner dependent upon DC numbers, Ag concentrations, and culture periods (Fig. 2*A*). Importantly, DC failed to activate DO11.10 T cells in the absence of OVA peptide. Considering that FCS in our culture medium contains many xenogenic proteins, we interpreted this to validate the Ag specificity of our in vitro Ag presentation assay. The availability of relatively large numbers of Ag-specific CD4$^+$ T cells exhibiting a naive phenotype (~1×10^7 cells/mouse) and DC showing a relatively immature phenotype (~1×10^7 cells/mouse) has allowed us to study in a systematic fashion the pharmacological activities of different immunosuppressants to regulate bidirectional intercellular communication between DC and T cells during Ag presentation.

Differential impacts on DC-induced T cell activation

To study the effects of immunosuppressive agents on DC-dependent activation of naive T cells, we first added each agent at graded concentrations to the "complete" cocultures containing bone marrow-derived DC, DO11.10 T cells, and OVA peptide. DC-induced T cell proliferation was suppressed most efficiency by RAP and FK506, with 25–75% inhibition achieved at 10^{-10}–10^{-8} M (Fig. 2*B*). The dose-response curve for CyA was almost superimposable on that for FK506, although CyA required ~50 times higher concentrations to achieve similar inhibition. DEX inhibited T cell proliferation only partially even at the highest concentration (10^{-5}

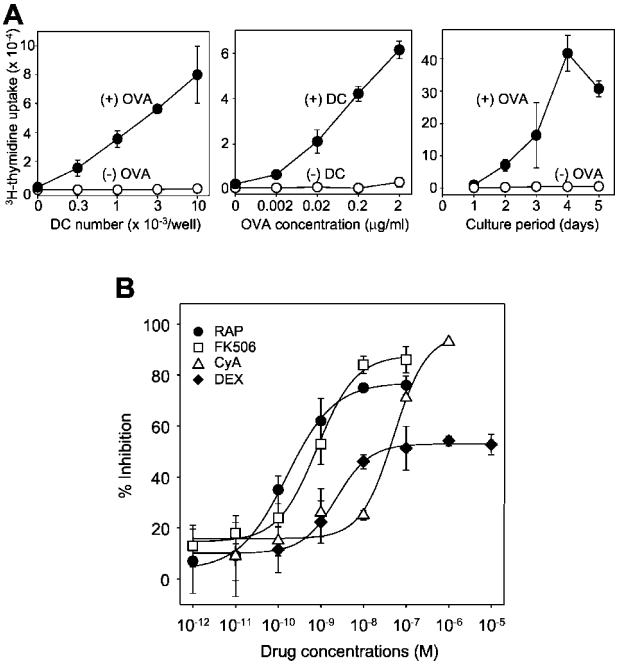

FIGURE 2. Effects of immunosuppressive agents on DC-induced, Ag-specific proliferation of DO11.10 T cells. *A*, DO11.10 T cells (2.5×10^5 cells/ml) were cultured with the indicated numbers of gamma-irradiated DC in the presence or the absence of 2 μg/ml OVA peptide (*left*), with the indicated concentrations of OVA peptide in the presence or the absence of DC (5×10^4 cells/ml; *middle*), or with DC (5×10^4 cells/ml) in the presence or the absence of 2 μg/ml OVA peptide (*right*). Data are representative of two independent experiments, showing the mean ± SD ($n = 3$) [^{3}H]thymidine uptake on day 3 (*left* and *middle*) or at the indicated time points (*right*). *B*, Each reagent was added at the indicated concentrations to the complete cocultures of DC (5×10^4 cells/ml), T cells (2.5×10^5 cells/ml), and OVA peptide (2 μg/ml). Data are representative of three independent experiments, showing the mean ± SD ($n = 3$) percent inhibition of [^{3}H]thymidine uptake on day 3.

M). These results indicate a quantitative difference among the immunosuppressants in their relative efficacies to inhibit DC-dependent T cell proliferation.

DO11.10 T cells produced IFN-γ, IL-2, and IL-4 upon Con A stimulation, whereas bone marrow-derived DC secreted IL-6 and IL-12 p40 in response to LPS treatment (data not shown). These results suggested their potential to secrete respective cytokines during Ag presentation. In fact, all the above cytokines were detected by ELISA in the supernatants from the complete cocultures (Fig. 3*A*). By contrast, none of these cytokines was detected at significant levels in incomplete cocultures lacking any single component. To identify the relevant cell type responsible for the production of each cytokine, we next examined intracellular cytokine deposition by FACS. When T cells and DC were cocultured in the presence of OVA peptide, intracellular accumulation of IL-2 was observed in 15.2% of the CD4$^+$ populations, whereas no IL-2-containing CD4$^+$ cells were detected in the absence of Ag (Table I). By contrast, only minor fractions (1.7%) of the CD11c$^+$ population exhibited intracellular IL-2. Likewise, relatively small, but significant, numbers of CD4$^+$ cells, but not CD11c$^+$ cells, expressed intracellular accumulation of IL-4 and IFN-γ only in the complete coculture. Although the frequencies of those cytokine-producing T cells may appear rather low, our findings are in complete agreement with the previous report that only limited fractions of DO11.10 T cells express mRNA for the above cytokines upon stimulation (44). Conversely, intracellular deposition of IL-6 and IL-12 p40 became detectable almost exclusively within the CD11c$^+$ populations in the complete coculture, but not in the incomplete coculture lacking OVA peptide. Thus, IL-2, IL-4, and IFN-γ are produced primarily by T cells in our in vitro Ag presentation system, whereas DC serve as the main source for IL-6 and IL-12 p40. Our observations also highlight the bidirectionality of Ag-specific DC-T cell interaction, with DC triggering the production of selected cytokines by naive T cells, while T cells inducing the secretion of different cytokines by DC.

In three independent experiments, FK506 was significantly more potent than any other agent in inhibiting DC-triggered production of IFN-γ, IL-2, and IL-4 by T cells (Fig. 3*B*, *upper panels*). All three cytokines were inhibited by FK506 uniformly at similar concentrations (10^{-10}–10^{-8} M). DEX was the second most potent, although it inhibited IL-2 secretion only partially even at 10^{-5} M. Again, CyA required 30–50 times higher concentrations than did FK506 to inhibit each cytokine. Importantly, RAP even at the highest concentration (10^{-7} M) failed to markedly suppress the production of any cytokine by T cells. None of the drugs at the tested doses affected the viability of T cells, as tested by PI uptake (Fig. 3*B*, *upper right panel*).

DO11.10 T cells began to express CD69 after 20-h coculture with DC and OVA peptide (Fig. 4*A*, *left panels*). Conversely, CD40 expression by DC was up-regulated after coculture with T cells and Ag (Fig. 4*A*, *right panels*). Neither of these phenotypic changes was observed in any incomplete coculture. These results again highlight the bidirectionality of DC-T cell interaction, with DC triggering CD69 expression by T cells, while T cells induce CD40 up-regulation by DC. In two independent experiments, FK506 (10^{-9}–10^{-8} M) caused significant, albeit incomplete, inhibition of DC-induced CD69 expression by T cells (Fig. 4B, *left panel*). CyA required 30–50 times higher concentrations to induce similar inhibition. By contrast, no apparent inhibition of CD69 expression was achieved with RAP (up to 10^{-7} M) or DEX (up to 10^{-5} M). Again, none of the drugs at the tested doses affected the viability of T cells (Fig. 4*C*).

Differential impacts on T cell-induced DC maturation

As described above, DC produce IL-6, IL-12 p40, and IL-12 p70 upon Ag-specific interaction with T cells (Fig. 3*A*). In two independent experiments FK506 was significantly more potent than any other agent in inhibiting DC cytokine production (Fig. 3*B*, *lower panels*). All three cytokines (IL-6, IL-12 p40, and IL-12 p70) were inhibited by FK506 efficiently (up to 80–100%) and uniformly at similar concentrations (10^{-10}–10^{-8} M). CyA also inhibited all three cytokines, albeit at 30–50 times higher concentrations. The extent of DEX-induced suppression was relatively incomplete (50–60%) even at 10^{-5} M. RAP at the tested concentrations (up to 10^{-7} M) caused only marginal, if any, inhibition of cytokine production by DC. None of the drugs at the tested concentrations significantly affected the viability of DC as examined by PI uptake (Fig. 3*B*, *lower right panel*).

Bone marrow-derived DC elevate CD40 expression during Ag presentation (Fig. 4*A*, *right panel*). RAP inhibited this T cell-induced CD40 up-regulation by DC most efficiently (at 10^{-10}–10^{-9} M) and most completely (50–100% inhibition; Fig. 4*B*, *right panel*). Other agents required significantly higher concentrations (10^{-9}–10^{-8} M for FK506 and 10^{-7}–10^{-6} M for CyA and DEX) to suppress CD40 up-regulation. Again, none of the agents at the tested doses affected the viability of DC (Fig. 4*C*). We also observed elevated expression of I-A and CD86 by DC in the complete coculture containing OVA peptide compared with the incomplete coculture lacking Ag (Fig. 5). Each of the four agents in the

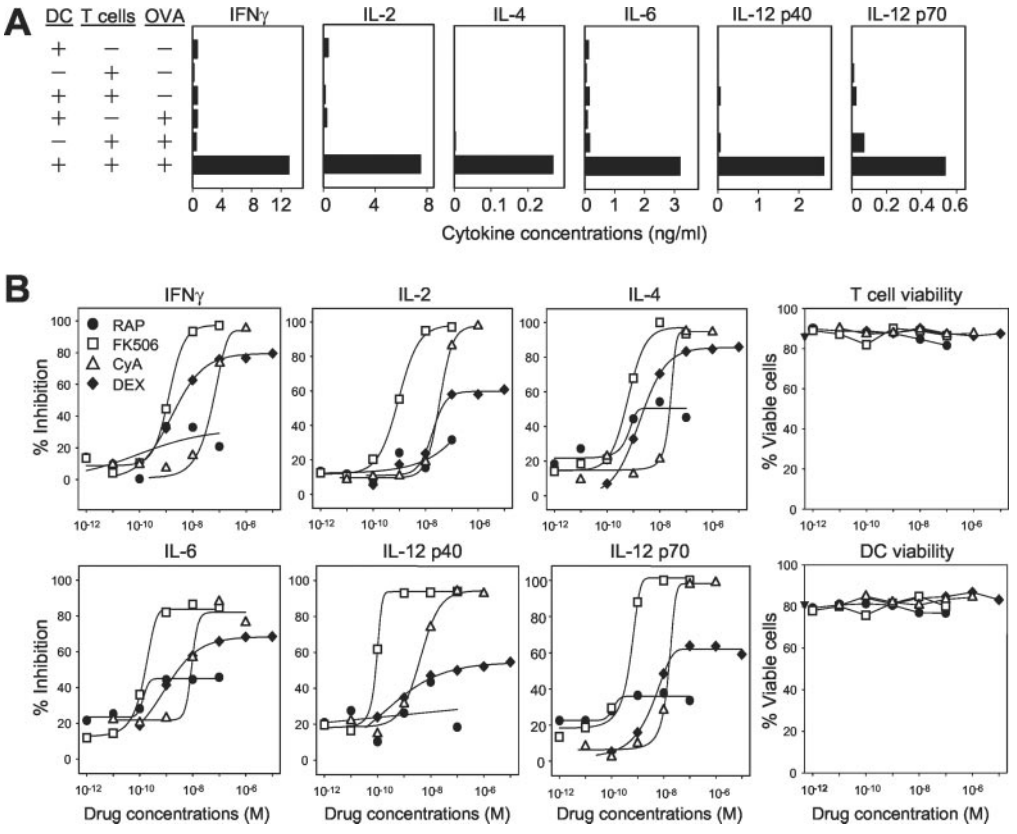

FIGURE 3. Effects of immunosuppressive agents on cytokine secretion during Ag presentation. *A*, Bone marrow-derived DC and/or DO11.10 T cells were cultured for 20 h in the presence or the absence of OVA peptide. Culture supernatants were tested for the indicated cytokines by ELISA. *B*, Each reagent was added at the indicated concentrations to the complete cocultures. Culture supernatants were tested for the indicated cytokines by ELISA. Cell viability of DC or T cells was assessed by PI uptake by $CD11c^+$ or $KJ1.26^+$ populations, respectively. ▼, Baseline cell viabilities in the absence of added drugs. The data shown in this figure are representative of three independent experiments.

above concentration ranges was found to inhibit this T cell-dependent I-A and CD86 expression by DC without causing significant DC death. Taken together, our observations illustrate quantitative and qualitative differences among the four immunosuppressants in their pharmacological activities to inhibit various aspects of T cell-dependent DC maturation.

To determine whether any of the agents would cause apoptotic changes in DC and/or T cells during Ag presentation, we examined the surface expression of phosphatidylserine as an early apoptotic phenotype. Briefly, after 24-h coculture of T cells and DC in the presence of each immunosuppressant, we counted the numbers of annexin V-positive/PI-negative cells within the $KJ1.26^+$ population and $CD11c^+$ population (Table II). The presence of OVA peptide in the coculture did not alter the number of such apoptotic cells in either population, suggesting that T cell activation signals or DC maturation signals do not directly initiate apoptotic processes in our experimental system. Addition of RAP (10^{-8} M) or CyA (10^{-6} M) caused a significant, albeit modest (~5%), increase in the number of apoptotic DC, but not in the number of apoptotic T cells, corroborating the previous report that RAP at the same concentration induced externalization of phosphatidylserine in ~10% of human DC in 24 h (24). In summary, although none of the agents caused significant death of DC or T cells (as measured by PI uptake), RAP and CyA induced an early apoptotic change in relatively small fractions of DC during Ag presentation.

Table I. *Production of different sets of cytokines by T cells and by DC during Ag presentation*[a]

	T Cells		DC	
Cytokine	− OVA	+ OVA	− OVA	+ OVA
IL-2	0.0 ± 0.0	15.2 ± 0.5[b]	0.2 ± 0.2	1.7 ± 0.6[c]
IL-4	0.2 ± 0.0	0.7 ± 0.1[b]	0.2 ± 0.1	0.6 ± 0.3
IFN-γ	0.2 ± 0.0	1.3 ± 0.1[b]	0.2 ± 0.1	0.3 ± 0.1
IL-6	0.1 ± 0.1	0.1 ± 0.1	0.8 ± 0.4	4.0 ± 0.8[b]
IL-12	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2	18.7 ± 0.9[b]

[a] Bone marrow-derived DC and DO11.10 T cells were cocultured for 22 h in the presence or the absence of OVA peptide. Samples were then labeled with FITC-conjugated anti-CD4 or anti-CD11c mAb, followed by intracellular staining for the indicated cytokines. Data are representative of two independent experiments, showing the mean ± SD ($n = 3$) percentage of cytokine-positive cells within the $CD4^+$ or $CD11c^+$ populations.

[b] $p < 0.01$ compared to the control samples cultured in the absence of OVA peptide.

[c] $p < 0.05$ compared to the control samples cultured in the absence of OVA peptide.

Impact on LPS-triggered DC maturation

To determine whether any of these immunosuppressants would inhibit the maturational changes in DC in the absence of T cells, we next stimulated bone marrow-derived DC with LPS. LPS treatment triggered the secretion of IL-6 (61.6 ng/ml with LPS vs <0.1 ng/ml without LPS) and IL-12 p40 (42.9 vs <0.1 ng/ml), and it also up-regulated CD40 expression (with mean fluorescence intensity values of 651 vs 58). As shown in Fig. 6, DEX at 10^{-8}–10^{-7} M caused partial (~50%) inhibition of LPS-induced IL-6 and IL-12 p40 production, corroborating the previous report (21). By contrast, other drugs failed to inhibit cytokine production by LPS-stimulated DC. Moreover, none of the agents efficiently abolished

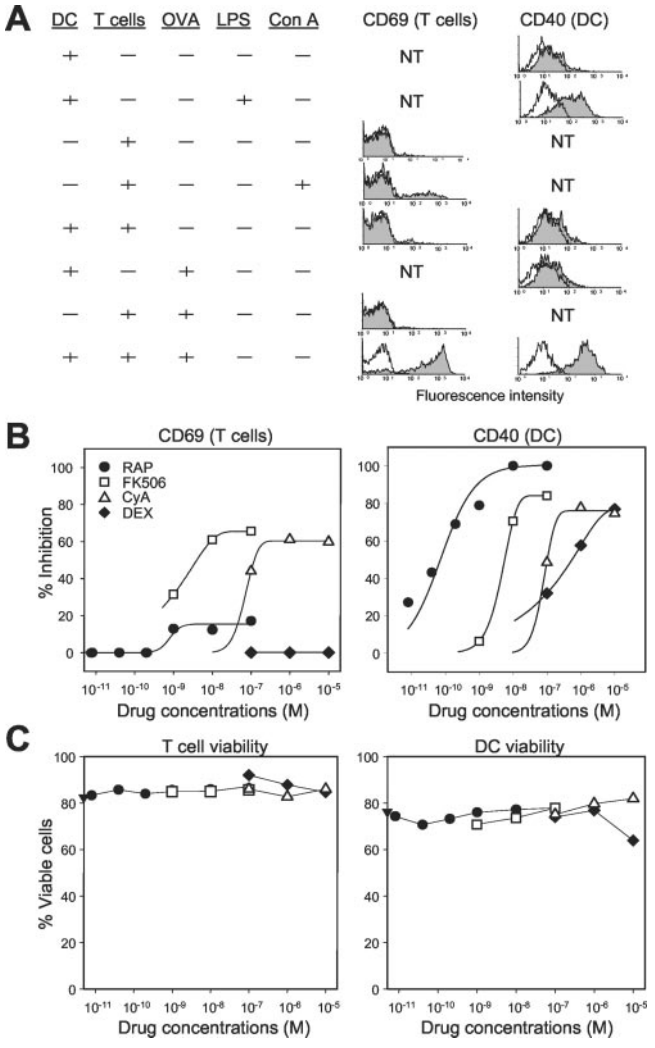

FIGURE 4. Effects of immunosuppressive agents on CD69 expression by T cells and CD40 expression by DC during Ag presentation. *A*, Bone marrow-derived DC and/or DO 11.10 T cells were cultured for 20 h in the presence or the absence of LPS, Con A, or OVA peptide and then examined for surface expression of CD69 by the KJ1.26$^+$ T cell populations and CD40 by the CD11c$^+$ DC populations (filled histogram). The staining profiles with isotype-matched control IgG (open histogram). *B*, Each reagent was added at the indicated concentrations to the complete cocultures. Samples were then examined for CD69 expression by T cells and CD40 expression by DC. *C*, Cell viability of DC or T cells was assessed by PI uptake by CD11c$^+$ or KJ1.26$^+$ populations, respectively. ▼, The baseline cell viabilities in the absence of added drugs. Data are representative of two independent experiments, showing the percent inhibition of the mean fluorescence intensity (MFI).

LPS-induced CD40 expression by DC. Thus, maturational changes that occur in DC as a consequence of Ag-specific interaction with T cells are more susceptible to RAP and calcineurin inhibitors than are the same changes induced by LPS in the absence of T cells.

Differential impacts on secondary DC-T cell interaction

To test potential effects on the secondary T cell activation, we generated Th1 and Th2 cells from DO11.10 T cells. The resulting T cell populations both proliferated vigorously when restimulated with bone marrow-derived DC and OVA peptide. Proliferative responses of both Th1 and Th2 cells were inhibited most efficiently by FK506 and RAP, with 30–70% inhibition observed at 10^{-10}–10^{-9} M (Fig. 7*A*). CyA and DEX were significantly less potent in suppressing DC-induced proliferation of primed T cells.

Upon restimulation with DC and OVA peptide, Th1 cells secreted relatively large amounts of IFN-γ and IL-2, while Th2 cells secreted IL-4 (Fig. 7*B*, *left panels*). In the absence of Ag, neither Th1 nor Th2 cells secreted any cytokine in significant levels, indicating their Ag specificity. Consistent with our observations with naive T cells, FK506 (10^{-8} M) and CyA (10^{-6} M) almost completely inhibited DC-induced IFN-γ and IL-2 production by Th1 cells as well as IL-4 production by Th2 cells. By contrast, cytokine production by either Th1 or Th2 cells was inhibited only minimally by RAP (10^{-8} M) or DEX (10^{-7} M).

Conversely, Th1 cells, but not Th2 cells, triggered IL-12 p40 and p70 secretion by DC in an Ag-specific manner (Fig. 7*B*, *right panels*). This IL-12 production was inhibited almost completely by FK506 (10^{-8} M) or CyA (10^{-6} M) and only marginally by DEX (10^{-7} M). In two independent experiments RAP (10^{-8} M) failed to significantly suppress Th1-induced IL-12 production by DC, as we observed with naive T cells. These observations illustrate contrasting effects of RAP and calcineurin inhibitors on DC maturation during secondary T cell activation.

Discussion

We compared four standard immunosuppressive agents systematically for their pharmacological impact on DC-induced T cell activation using an in vitro Ag presentation system. As summarized in Fig. 8, FK506 at 10^{-10}–10^{-8} M inhibited uniformly all tested aspects of DC-induced activation of naive T cells, including proliferation and secretion of IFN-γ, IL-2, and IL-4. Likewise, the second calcineurin inhibitor CyA also uniformly inhibited all these changes, although requiring 30–50 times higher concentrations. By marked contrast, RAP at 10^{-10}–10^{-8} M inhibited DC-induced T cell proliferation efficiently, while it failed to abrogate cytokine production even at 10^{-7} M. These findings may first appear to simply mirror the previous reports describing the effects of RAP vs calcineurin inhibitors on T cell activation triggered by mitogenic lectins, phorbol ester plus calcium ionophore, or anti-CD3 mAb plus anti-CD28 mAb (11–16). However, it should be emphasized that this is the first report documenting their contrasting influences on DC-dependent, Ag-specific T cell activation. We have also identified an additional qualitative difference; FK506 and CyA, but not RAP, suppressed DC-induced CD69 up-regulation by DO11.10 T cells during Ag presentation. Vanasek et al. (45) reported very recently that RAP treatment prevents OVA peptide-triggered clonal expansion of adoptively transferred DO11.10 T cells without affecting their induced CD69 expression in living animals (although direct roles played by DC were not addressed). Taken together, these observations document a unique pharmacological property of RAP to selectively inhibit T cell proliferation without affecting other T cell activation-associated changes.

DC are known to undergo a series of maturational changes upon Ag-specific interaction with T cell clones or ligation of surface CD40 (25–31). On the other hand, it remains relatively unclear whether naive T cells induce similar maturation changes in DC during Ag presentation. Upon Ag-specific interaction with DO11.10 T cells, bone marrow-derived DC elevated their surface expression of CD40, I-A, and CD86 and produced IL-6, IL-12 p40, and IL-12 p70. Not only do these observations suggest the ability of naive T cells to trigger DC maturation, they have also formed the basis for our subsequent analyses to determine the impact of immunosuppressive drugs on T cell-dependent DC maturation. We observed that FK506 at 10^{-10}–10^{-8} M uniformly inhibited CD40 up-regulation by DC as well as their production of IL-6, IL-12 p40, and IL-12 p70 (Fig. 8). CyA and DEX also inhibited all these changes at higher concentrations. Strikingly, RAP at 10^{-10}–10^{-9} M completely inhibited CD40 expression by DC, whereas it failed

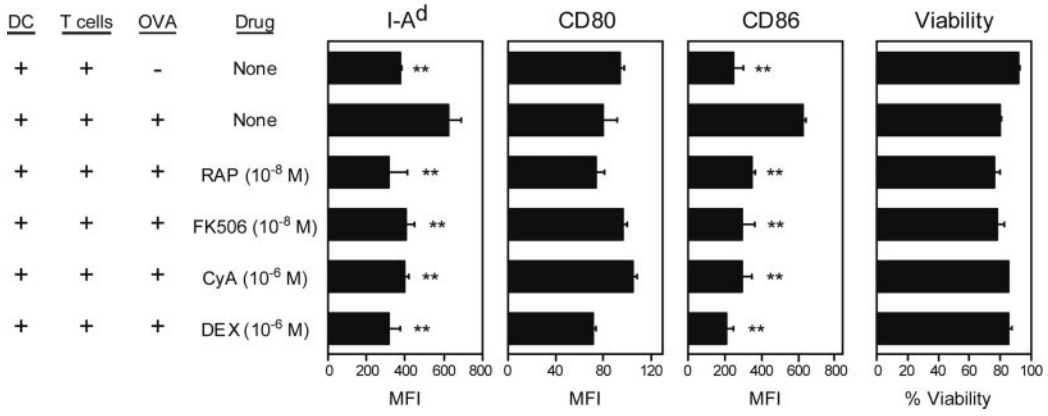

FIGURE 5. Effects of immunosuppressive agents on phenotypic maturation of DC during Ag presentation. Bone marrow-derived DC and DO11.10 T cells were cocultured for 20 h in the presence or the absence of OVA peptide and/or the indicated immunosuppressants. Samples were then examined for surface expression of I-A, CD80, and CD86 expression and for PI uptake within the $CD11c^+$ populations. Data are representative of two independent experiments, showing the means ± SD mean fluorescence intensity (MFI) and percent cell viability from triplicate cultures. Asterisks indicate statistically significant differences ($p < 0.01$) compared with the complete coculture (containing OVA peptide) in the absence of added agents.

to markedly abolish their production of any cytokine even at 10^{-7} M. These observations introduce a new concept that calcineurin inhibitors broadly block DC→T cell activation and T cell→DC maturation signals being exchanged between DC and T cells during Ag presentation, whereas RAP exhibits a highly selective inhibitory profile to block rather limited aspects.

FK506 was indistinguishable from CyA in its overall suppressive profiles, although FK506 was 30–50 times more potent in each parameter (Fig. 8). These observations are in agreement with the notion that the two drugs block the same molecular targets (i.e., calcineurin and its substrates) and, thus, share therapeutic outcomes and adverse effects (3, 4). The pharmacological dose ranges we have identified for FK506 and CyA in our in vitro Ag presentation system correspond closely to the known therapeutic trough blood levels of 5–15 ng/ml ($0.6–1.8 \times 10^{-8}$ M) for FK506 and 150–300 ng/ml ($1.3–2.5 \times 10^{-7}$ M) for CyA (46). Likewise, RAP inhibited DC-dependent T cell proliferation and T cell-triggered CD40 up-regulation by DC in the dose range corresponding to its known therapeutic blood levels of 5–15 ng/ml ($0.5–1.5 \times 10^{-8}$ M) (47).

Several features that distinguish RAP from calcineurin inhibitors have been observed in animal models of immunological diseases. For example, Bundick et al. (48) observed that RAP inhibited the induction of Th2-polarized chronic GVHD in the (C57BL/$6 \times$ DBA/2)F_1 recipients after repeated administrations of parental DBA/2 spleen cells, whereas FK506 and CyA at relatively low doses enhanced disease development. Although these authors interpreted their in vivo observations to suggest that calcineurin inhibitors may selectively suppress Th1-mediated immune responses, our in vitro data failed to support this concept because calcineurin inhibitors and RAP effectively inhibited DC-dependent activation of both Th1 and Th2 subsets. Sehgal's group (49, 50) reported that spontaneous progression of systemic lupus erythematosus in MRL/1 mice was prevented by RAP, but not by CyA. Interestingly, disease development in the above model was closely

Table II. *Early apoptotic changes in T cells and DC during Ag presentation in the presence of immunosuppressive agents*[a]

OVA	Drug	% Apoptotic Cells	
		T cells	DC
−	None	2.1 ± 0.03	6.4 ± 1.8
+	None	3.2 ± 1.0	6.3 ± 1.6
+	RAP (10^{-8} M)	4.6 ± 0.6	10.3 ± 0.4[b]
+	FK506 (10^{-8} M)	5.2 ± 1.2	9.8 ± 2.3
+	CyA (10^{-6} M)	4.8 ± 0.3	10.0 ± 1.7[b]
+	DEX (10^{-6} M)	3.5 ± 0.4	5.9 ± 0.9

[a] Bone marrow-derived DC and DO11.10 T cells were cocultured for 24 h in the presence or the absence of OVA peptide and/or the indicated agents. Samples were then examined for PI uptake and annexin V binding within the $KJ1.26^+$ population (*left*) and the $CD11^+$ population (*right*). Data are representative of two independent experiments, showing the means ± SD ($n = 3$) percentage of annexin V-positive/PI-negative cells.

[b] Statistically significant ($p < 0.05$) differences compared to the control complete cocultures containing OVA peptide, but not any immunosuppressive agents.

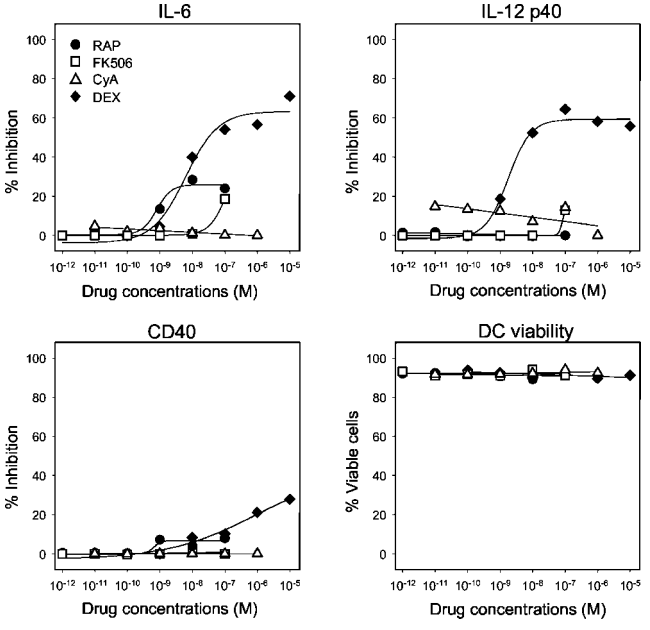

FIGURE 6. Effects of immunosuppressive agents on LPS-induced DC maturation. $CD11c^+$ populations (>99% pure) sorted from bone marrow-derived DC cultures (day 5) were incubated for 20 h in the presence or the absence of LPS (100 ng/ml) and/or the indicated immunosuppressants. The cells were then examined for the secretion of the indicated cytokines by ELISA, surface expression of CD40, and PI uptake. Data are representative of two independent experiments, showing the percent inhibition compared with the positive control DC that were stimulated with LPS in the absence of added agents.

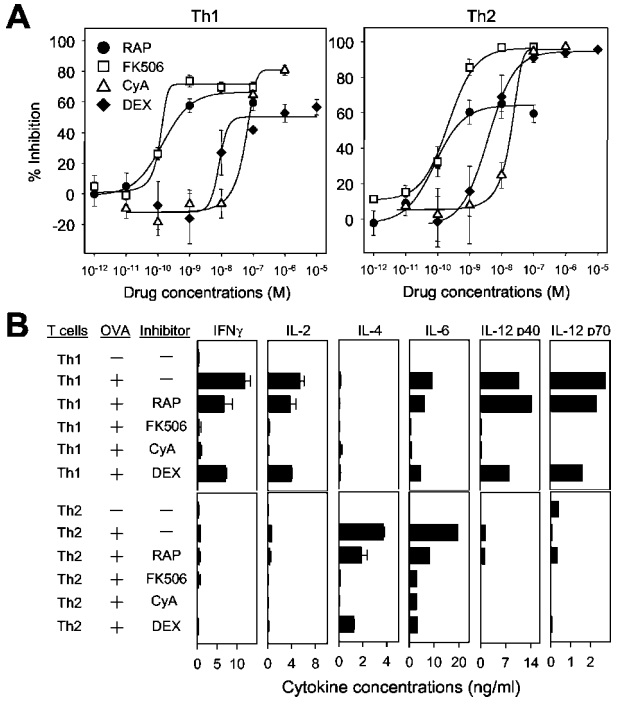

FIGURE 7. Effects of immunosuppressive agents on DC interaction with primed Th1 and Th2 subsets. *A*, OVA-reactiveTh1 cells (*left*) or Th2 cells (*right*) were cultured with DC and $OVA_{323–339}$ peptide in the presence of each immunosuppressive reagent added at the indicated concentrations. Data are representative of two independent experiments, showing the mean ± SD (n = 3) percent inhibition of [^{3}H]thymidine uptake on day 2. *B*, The same Th1 or Th2 cells were cocultured with DC and/or OVA peptide for 20 h in the presence or the absence of RAP (10^{-8} M), FK506 (10^{-8} M), CyA (10^{-6} M), or DEX (10^{-7} M). Culture supernatants were then tested for the indicated cytokines by ELISA. Data shown are representative of two independent experiments.

associated with the severely impaired ability of T cells to secrete IL-2 in response to mitogenic stimulation, and this ability was restored only in those animals treated with RAP. It is, therefore, tempting to speculate that the observed prophylactic activity of RAP may be explained in part by its unique pharmacological activity to inhibit DC-induced T cell activation without markedly affecting IL-2 production. Vogelsang and Hess (51) reported that administration of CyA, but not RAP, induced GVHD-like autoimmune reactions in the recipients of syngeneic bone marrow transplantation. Although mechanisms underlying the above opposing effects remain unclear, only those animals receiving CyA showed a marked increase in the number of autoreactive T cells (as defined by Vβ8 TCR expression), consistent with other reports that calcineurin inhibitors, but not RAP, affect negative thymic selection of autoreactive T cells (52, 53). Thus, it will be interesting to determine whether RAP may differ from calcineurin inhibitors in the impact on thymic DC, as we have observed with bone marrow-derived DC. Wang et al. (54) reported that CyA prevented allogeneic heart graft rejection only when administered from the day of transplantation, whereas RAP treatment was effective even when started 4 days after transplantation. Likewise, RAP and CyA both showed significant prophylactic and therapeutic efficacies to suppress the progression of adjuvant arthritis, whereas rebound of the disease state was observed after discontinuation of CyA, but not RAP (50, 55). These differences may be explained at least partially by the current idea that CyA efficiently inhibits early events (e.g., cytokine production and cytokine receptor expression) in DC-induced activation of naive T cells, whereas RAP inhibits subsequent events, including cytokine-mediated expansion of activated T cells. Alternatively, the unique inhibitory impact of RAP on T cell-dependent DC maturation (i.e., blocking CD40 expression without abrogating their cytokine production) may explain a

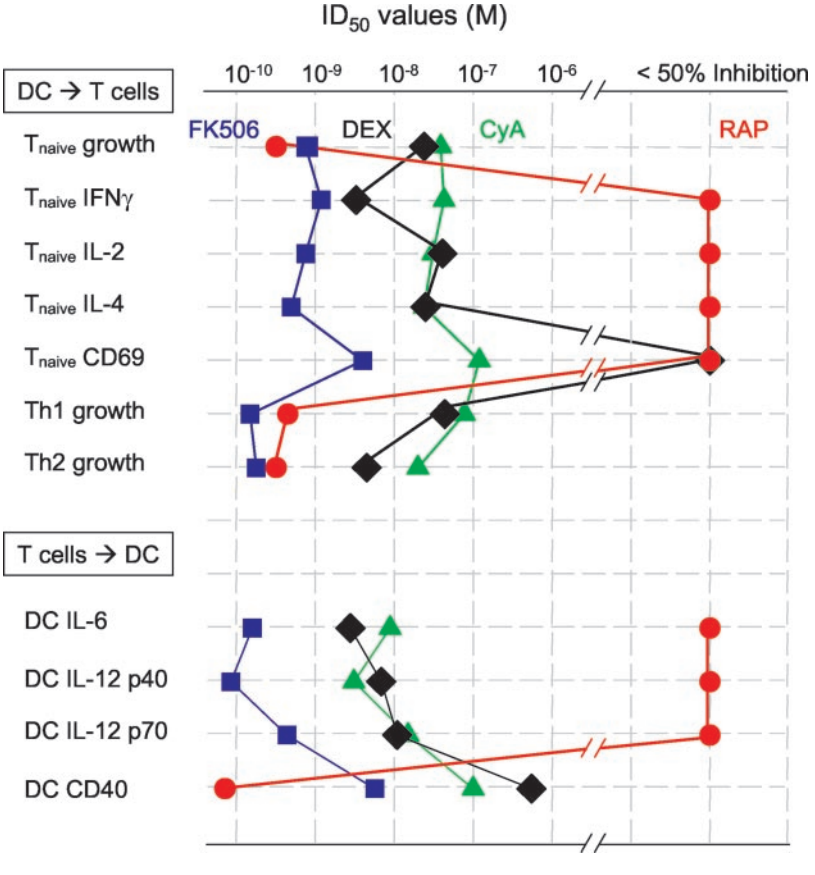

FIGURE 8. Overall impacts of the four tested immunosuppressive agents on Ag presentation. The 50% inhibitory doses (ID_{50}) were calculated for each agent from the representative dose-dependency curves shown in Figs. 2*B*, 3*B*, 4*B*, and 7*A*. The ID_{50} values are plotted for the indicated parameters of bidirectional DC-T cell interaction. The parameters that were inhibited only partially (<50%) at the highest tested concentrations (10^{-7} M RAP and 10^{-5} M DEX) are plotted on the *right column* and designated <50% inhibition.

causative mechanism by which RAP induces long-lasting tolerance. In this regard, Li et al. (56) reported recently that stable acceptance of allogeneic skin grafts is achievable by a combination of RAP and costimulation blockers (CTLA4-Ig plus anti-CD40L mAb), but not by a combination of CyA and the same blockers. They postulated that costimulation blockers alone reduce the frequency of proliferating allo-reactive T cells, while added RAP promotes apoptosis of such proliferating T cells via activation-induced cell death. By contrast, the combination of the costimulation blockers and CyA inhibits both signal 1 and signal 2, leading to complete inhibition of proliferation and apoptosis of allo-reactive T cells. Although neither RAP nor CyA showed a significant effect on DC-induced T cell apoptosis in our system, it will be interesting to determine whether contrasting effects on T cell apoptosis may be revealed by addition of the costimulation blockers in the cocultures of DC and T cells. In summary, RAP clearly differs from calcineurin inhibitors in their in vivo immunoregulatory profiles, which may be explained by our observation that RAP differs from CyA and FK506 in their in vitro pharmacological profiles to inhibit bidirectional DC-T cell communication in Ag presentation.

Acknowledgments

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