Rapamycin inhibits macropinocytosis and mannose receptor–mediated endocytosis by bone marrow–derived dendritic cells

Holger Hackstein, Timucin Taner, Alison J. Logar, and Angus W. Thomson

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that use 2 major pathways for antigen uptake: constitutive macropinocytosis and mannose receptor–mediated endocytosis. Efficient endocytosis is critical for DCs to fulfill their sentinel function in immunity. We investigated the influence of the immunosuppressive macrolide rapamycin on macropinocytosis of fluorescein isothiocyanate (FITC)–albumin and mannose receptor–mediated endocytosis of FITC-dextran by murine bone marrow–derived DCs by flow cytometry. The data show that (1) at a low, physiologically relevant concentration (1 ng/mL), rapamycin impairs macropinocytosis and mannose receptor–mediated endocytosis; (2) the effects are independent of DC maturation and can be demonstrated specifically in immature CD11c$^+$ major histocompatibility complex (MHC) class II^{lo} DCs by 3-color flow cytometry; (3) inhibition of endocytosis is not related to apoptotic cell death; and (4) molar excess of the structurally related molecule FK506 inhibits the actions of rapamycin. The inhibitory effects of rapamycin on DC endocytosis were confirmed in vivo. To our knowledge, this is the first report that a clinically relevant immunosuppressant inhibits DC endocytosis. (Blood. 2002;100:1084-1087)

Introduction

Dendritic cells (DCs) arise from CD34$^+$ bone marrow (BM) stem cells and represent a heterogenous population of ubiquitously distributed antigen-presenting cells (APCs) that play critical roles as initiators and modulators of immune responses.[1,2] Among the most striking features underlying the efficiency of DCs as APCs is their unsurpassed capacity to take up antigens via constitutive macropinocytosis and mannose receptor–mediated endocytosis[3] and to subsequently process and present major histocompatibility complex (MHC)–antigen complexes on their surface.[1] The capacity of DCs to endocytose and to present antigens is under tight developmental control: immature DCs are excellent at internalizing antigens but express low surface levels of MHC class II molecules, whereas mature DCs down-regulate endocytotic activity and up-regulate MHC class II and costimulatory molecules (CD40, CD80, CD86) that promote T-cell activation.[1] Anti-inflammatory drugs such as corticosteroids[4] or salicylates[5] suppress DC maturation and as a consequence enhance their endocytotic activity. Recent reports point toward Rho family proteins Cdc42[6] and Rac,[7] as well as aquaporins,[8] as important factors that regulate DC endocytosis. Rapamycin is a potent immunosuppressive macrolide, isolated from *Streptomyces hygroscopicus*, that inhibits downstream signaling from the targets of rapamycin proteins (TORs) by forming a complex with its intracellular receptor FK506-binding protein 12 (FKBP12) and TORs.[9] It is used clinically to prevent and treat allograft rejection.[10-12] Interaction of the rapamycin-FKBP12 complex with TORs results in inhibition of multiple biochemical pathways (eg, p70 S6 kinase, cyclin-dependent kinases, translational effector proteins) that are critical for cytokine/growth factor–induced cellular proliferation, ribosome biosynthesis, translation initiation, and cell cycle progression into S phase.[9,13] In view of the paucity of information concerning the influence of rapamycin on APC function and its well-documented inhibitory effects on protein synthesis, we analyzed the impact of rapamycin on DC endocytosis. Our results indicate that rapamycin is an inhibitor of DC endocytosis in vitro and in vivo and that molar excess of the structurally related immunophilin ligand FK506 partially reverses its inhibitory effects.

Study design

Generation of BM-derived DCs

Male C57BL/10J (I-A^b) mice, 8 to 12 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME). BM-derived DCs were generated and characterized as described,[5] with minor modifications. Briefly, BM cells were cultured for 7 days in RPMI-1640 with 10% heat-inactivated fetal calf serum (FCS), L-glutamine, nonessential amino acids, sodium pyruvate, penicillin-streptomycin, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2-mercaptoethanol (2-ME) (all from Life Technologies, Gaithersburg, MD), 1000 U/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Kenilworth, NJ) ± 1000 U/mL murine interleukin 4 (IL-4) (R&D Systems, Minneapolis, MN). At day 2, 1-100 ng/mL rapamycin (Sigma, St Louis, MO) ± 20-250 ng/mL FK506 (Prograf for intravenous use, Fujisawa Healthcare, Deerfield, IL) was added. Every 2 days, 75% supernatant was

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replaced with fresh cytokine-containing medium (± rapamycin, FK506). On day 4, nonadherent cells were removed; on day 7, ≥ 50% of the nonadherent cells expressed CD11c.

In vivo administration of rapamycin

Rapamycin (Wyeth-Ayerst, Princeton, NJ) was dissolved in 51% wt/vol polyethylene glycol 300 (PEG300), 2.5% wt/vol polysorbate 80, 10% vol/vol ethanol (vehicle; reagents from Sigma). Chinese hamster ovary (CHO) cell-derived rhuman Flt3 ligand (10μg/kg/d, intraperitoneally; Immunex, Seattle, WA) was used to expand DCs in mice over a period of 10 days.[14] Animals were injected with rapamycin (0.5 mg/kg/d, intraperitoneally) or vehicle at the same times. Spleens were disaggregated with fine scissors and passed through a nylon cell strainer (Falcon Becton Dickinson, NJ) to obtain a single-cell suspension. Erythrocytes were lysed with 0.83% wt/vol NH_4Cl.

Endocytosis

Quantitative analysis of endocytosis was performed as described,[5] with minor modifications. Cells (5×10^5) were incubated with 5 μg/mL FITC-albumin (Sigma) or 0.1 mg/mL FITC-dextran (MW 42000, Sigma) at either 37°C or 4°C for 60 minutes (for in vivo–expanded DCs, 500 μg/mL FITC-albumin and 1 mg/mL FITC-dextran for 40 minutes). Endocytosis was stopped by 2 washes in ice-cold 0.1% sodium azide/1% FCS/phosphate-buffered saline (PBS). Cells were stained for CD11c (HL3) and, in some experiments, for MHC class II expression (IA^b β-chain, 25-9-17) as described (monoclonal antibodies from BD PharMingen, San Diego, CA).[5]

Apoptotic cell death

Apoptosis was analyzed by staining of phosphatidylserine translocation with FITC–annexin-V in combination with the vital dye 7-AAD (BD PharMingen) and detection of DNA fragmentation by terminal deoxynucleotidyl transferase-mediated fluorescein–2′-deoxyuridine 5′-triphosphate (dUTP) labeling (TUNEL assay; Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Positive controls included deoxyribonuclease (DNase) treatment and ultraviolet C (UVC) irradiation (60 J/m^2) in the TUNEL and annexin-V assay, respectively. Cells were costained for CD11c expression and analyzed by means of an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL).

Statistics

Statistical analysis was performed with a 2-tailed Student *t* test. Normal distribution of values was proved by the Kolomogorov-Smirnov test.

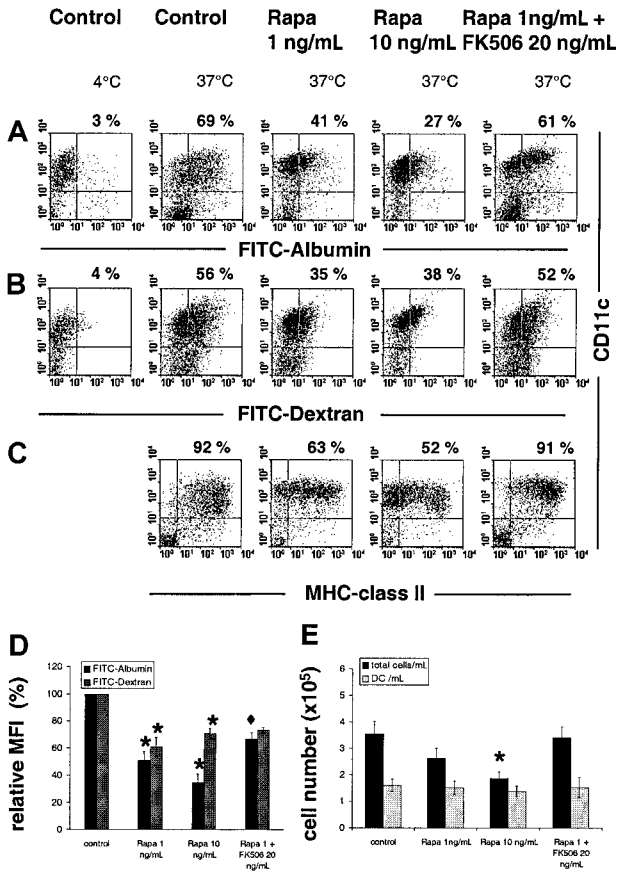

Figure 1. Rapamycin inhibits endocytosis by GM-CSF+IL-4–expanded DC. (A-D) BM-derived DCs were expanded for 7 days as described in "Study design"; rapamycin (Rapa) was added at day 2 (± FK506) at the concentrations indicated. FITC-albumin and FITC-dextran internalization at 37°C and 4°C (negative control), MHC class II (IA^b β-chain), and CD11c expression were analyzed by flow cytometry. In all experiments, $CD11c^+$ cells were analyzed to determine endocytosis and MHC class II expression specifically in DCs. (A-C) Numbers indicate the percentage of $CD11c^+$ cells that were positive for the marker indicated. (D) Data represent mean values (± SE) of $CD11c^+$ cells after subtraction of background fluorescence (4°C). (E) Comparison of total cell and DC numbers on day 7 of culture. (D, E) Differences between paired cultures were compared by 2-tailed Student *t* test for paired samples (*$P < .05$ vs control; ♦ $P < .05$ vs Rapa 1). (A-D) Results are representative of 4 (FITC-dextran) and 6 (FITC-albumin) separate experiments. (E) Results are representative of 6 separate experiments.

Results and discussion

Analysis of GM-CSF+IL-4–expanded, BM-derived DCs harvested at day 7 of culture revealed a reduced capacity of cells exposed to rapamycin to exhibit macropinocytosis of FITC-albumin and mannose receptor–mediated endocytosis of FITC-dextran. This was evident with respect to both the incidence (Figure 1A, B) and the mean fluorescence intensity (MFI; Figure 1D) of $CD11c^+$ cells. CD11c is a very reliable marker for murine DCs[15] and is not expressed in significant amounts by murine macrophages.[5,16] Because DC maturation is associated with marked down-regulation of endocytotic capacity,[3] we questioned whether these effects might be related to a stimulatory effect of rapamycin on DC maturation. However, rapamycin-exposed $CD11c^+$ DCs displayed significantly decreased surface MHC class II (Figure 1C) and costimulatory molecules (data not shown), indicating that they were more immature than untreated, control cells. It could be argued that the analysis of a fixed number of DCs by fluorescence-activated cell sorting (FACS) might mask an inhibitory effect of rapamycin on the differentiation of precursor cells into DCs. As shown in Figure 1E, however, rapamycin inhibited *total cell expansion* in a dose-dependent manner (antagonizable by a molar excess of FK506) but did not significantly block the *differentiation* of precursor cells into $CD11c^+$ DCs, as evidenced by the comparable yield of $CD11c^+$ DCs. Achievement of similar DC yields was due to consistently increased percentages of $CD11c^+$ DCs with characteristic DC morphology in the rapamycin-treated cultures.

Because additional experiments indicated that the inhibitory effect of rapamycin on DC maturation was IL-4 dependent (H.H., T.T., unpublished observations, October 2001), we expanded DCs with GM-CSF only. Generation of murine DCs in GM-CSF is a well-established culture method.[17] Additionally, to determine more precisely the endocytotic activity of homogenous DCs at the same stage of maturation, we specifically analyzed immature MHC II^{lo} DCs. These experiments confirmed the inhibitory effects of rapamycin on DC endocytosis and indicated that they were not IL-4 related (Figure 2A-C). Again, low concentrations of 1 ng/mL rapamycin (1.1 nmol) were sufficient to significantly and markedly suppress endocytotic activity. When 1 ng/mL rapamycin was used, the relative MFI of immature MHC class II^{lo} $CD11c^+$ DCs compared

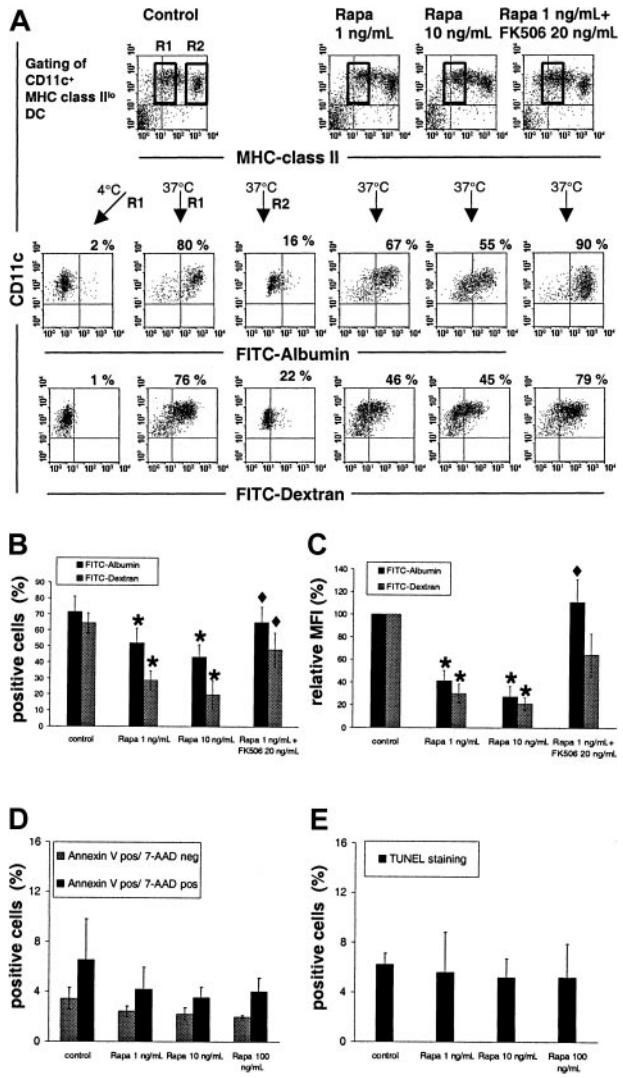

Figure 2. Rapamycin inhibits endocytosis by GM-CSF–expanded immature BM-derived DCs. (A-E) BM-derived DCs were expanded for 7 days with GM-CSF only, as described in "Study design"; rapamycin (Rapa) was added at day 2 (± FK506) at the concentrations indicated. (A-C) FITC-albumin and FITC-dextran internalization at 37°C and 4°C (negative control), MHC class II (IA^b β-chain), and CD11c expression were analyzed by 3-color flow cytometry. $CD11c^+$ MHC class II^{lo} cells were gated to determine endocytosis specifically in immature DCs. Regions R1 and R2 show endocytotic activity of immature MHC class II^{lo} and mature MHC class II^{hi} DCs, respectively. (A, B) Numbers indicate the percentage of $CD11c^+$ MHC class II^{lo} cells positive for the marker indicated. (B, C) Data represent mean values (± SE) of $CD11c^+$ MHC class II^{lo} cells after subtraction of background fluorescence (4°C). Differences between paired cultures were compared by the 2-tailed Student *t* test for paired samples (*$P \leq .01$ vs control; ♦ $P < .05$ vs Rapa 1). (D, E) Apoptosis of $CD11c^+$ DC was determined by annexin-V/7-AAD staining (D) and the TUNEL assay (E). Numbers represent mean values (± SE). Incidences of apoptotic (annexin-V positive/7-AAD negative or TUNEL positive) or secondary necrotic (annexin-V positive/7-AAD positive) cells were consistently lower than 10%. There was a trend toward *decreased* apoptosis/cell death in rapamycin-treated cultures, but the differences were not statistically significant. (A-C) Results are representative of 4 separate experiments. (D, E) Results are representative of 2 (TUNEL assay) and 3 (annexin-V/7-AAD assay) separate experiments.

with controls was < 42% and < 32% with respect to FITC-albumin and FITC-dextran, respectively (Figure 2C). Given that mean *trough* whole blood levels of rapamycin in patients are 17.3 ng/mL (5 mg rapamycin/d)[18] and that the free plasma fraction is 8%, these concentrations are clinically highly relevant. When rapamycin was added at day 6 of culture, it still inhibited DC endocytosis, but the overall effect was weaker (relative MFI 69% and 53% for FITC-albumin and FITC-dextran, respectively, at 5 ng/mL rapamycin).

Binding of rapamycin to FKBP12 and TOR inhibition can be antagonized in vitro by the structurally related immunophilin ligand FK506.[19,20] Whereas FK506 alone (20 ng/mL) did not interfere with DC endocytosis (data not shown), addition of 20 ng/mL FK506 to 1 ng/mL rapamycin (22.3-fold molar excess) antagonized the inhibitory effects of rapamycin, indicating that these were related to FKBP12-mediated TOR inhibition (Figures 1A, B; 2A-C). However, the antagonistic effect of FK506 was incomplete, especially with respect to mannose receptor–mediated endocytosis. Similar results were obtained over a wide range of drug concentrations (1-20 ng/mL rapamycin, 10-250 ng/mL FK506) and at different FK506/rapamycin ratios (10-55 molar). This suggested that other FKBPs might also be involved in endocytosis inhibition. One candidate is FKBP25, which has > 100 times greater binding affinity for rapamycin than FK506.[21]

Having established that rapamycin inhibited DC endocytosis, we analyzed whether this effect was due to increased apoptotic cell death. In contrast to a recent report,[22] the incidence of apoptosis at day 7 of culture was consistently low (< 10%) and was not affected significantly by rapamycin, even at a suprapharmacological dose of 100 ng/mL, as determined independently by annexin-V/7-AAD and TUNEL staining (Figure 2D, E). Similar results were obtained with GM-CSF+IL-4–expanded DCs and at day 4 of culture (data not shown). To investigate the in vivo relevance of DC endocytosis inhibition, we analyzed endocytotic activity in splenic DCs of animals that were injected with rapamycin (0.5 mg/kg/d for 10 days, ip) or vehicle and in which DCs were expanded with Flt3 ligand as we have described.[14] After 10 days, the animals were killed and FITC-albumin and FITC-dextran uptake by freshly isolated splenic $CD11c^+$ DCs was analyzed. The phenotype of

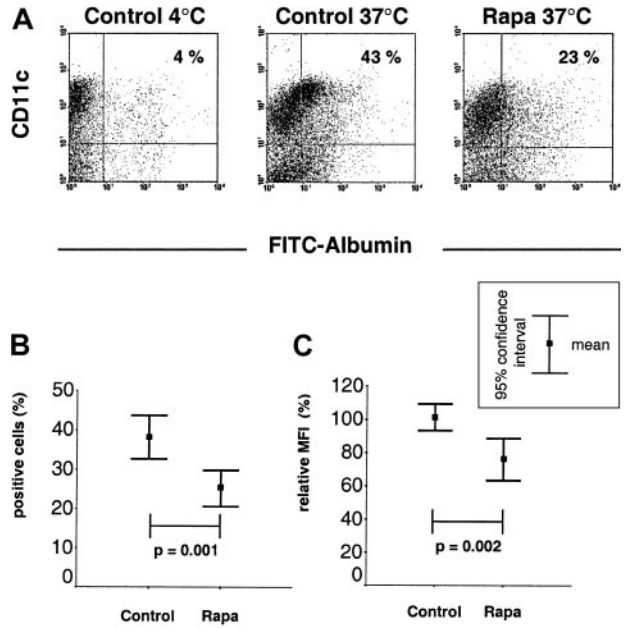

Figure 3. In vivo administration of rapamycin inhibits endocytosis by Flt3 ligand–expanded splenic DCs. (A-C) DCs were expanded in vivo with Flt-3 ligand and animals were injected with either rapamycin or vehicle, as described in "Study design." Endocytotic activity was measured by analyzing FITC-albumin uptake at 37°C and 4°C (negative control) and relative MFI in comparison with animals injected with vehicle only. Results are representative of 8 animals per group and were obtained in 5 independent experiments. Differences between the groups were compared by the 2-tailed Student *t* test for independent samples. Similar findings were obtained with respect to FITC-dextran uptake, as described in "Results and discussion."

these DCs was immature in both treatment groups. As shown in Figure 3, $CD11c^+$ DC of rapamycin-treated animals displayed significantly reduced macropinocytotic activity, with respect to both the number of positive cells and the relative MFI ($P = .001$ and $P = .002$, respectively). Similar findings were obtained with respect to FITC-dextran uptake (41.5% positive cells vs 24.8% in rapamycin-injected animals, $P < .05$; relative MFI 74.7%, $P < .05$).

The results of this study suggest that rapamycin can interfere with immune responses at a very early stage by inhibiting DC endocytosis. Thus, rapamycin targets a unique function of DCs that influences the induction of immunity against microbial pathogens (eg, *Salmonella*[23]) and allergens.[24] This effect may also suppress indirect alloantigen processing following transplantation.

The precise mechanisms by which rapamycin inhibits DC endocytosis remain to be determined. In this context, it is important to note that the Rho GTPases CDC42 and Rac that interfere with the endocytotic activity of DCs[7,8] complex with and activate the p70 S6 kinase[25] that belongs to the central signaling pathway disrupted by rapamycin.[9] In addition, rapamycin's inhibition of TOR signaling down-regulates protein translation and has been demonstrated to suppress actin synthesis.[26] Because, to our knowledge, no clinical immunosuppressant has been reported to inhibit DC endocytosis, these findings may provide further incentive for trials of rapamycin in clinical settings other than transplantation, for example, in autoimmune disease.

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