Everolimus Triggers Cytokine Release by Macrophages
Rationale for Stents Eluting Everolimus and a Glucocorticoid

Wim Martinet,* Stefan Verheye,* Inge De Meyer, Jean-Pierre Timmermans, Dorien M. Schrijvers, Ilse Van Brussel, Hidde Bult, Guido R.Y. De Meyer

Objective—Stent-based delivery of the mammalian target of rapamycin (mTOR) inhibitor everolimus is a promising strategy for the treatment of coronary artery disease. We studied potential adverse effects associated with mTOR inhibition.

Methods and Results—Macrophages in culture were either treated with everolimus or starved to inhibit mTOR. Everolimus led to inhibition of protein translation, activation of p38 MAPK, and the release of proinflammatory cytokines (eg, IL-6, TNFα) and chemokines (eg, MCP1, Rantes) before induction of autophagic death. These effects were also observed with rapamycin, but not after starvation. Everolimus-induced cytokine release was similar in macrophages lacking the essential autophagy gene Atg7 but was inhibited when macrophages were cotreated with p38 MAPK inhibitor SB202190 or the glucocorticoid clobetasol. Combined stent-based delivery of clobetasol and everolimus in rabbit plaques downregulated TNFα expression as compared with everolimus-treated plaques but did not affect the ability of everolimus to induce macrophage clearance.

Conclusion—mTOR inhibition by everolimus triggers cytokine release in macrophages through inhibition of protein translation and p38 activation. These findings provide a rationale for combined local treatment of atherosclerotic plaques with everolimus and an anti-inflammatory agent. (Arterioscler Thromb Vasc Biol. 2012;32:1228-1235.)

Key Words: atherosclerosis ■ macrophages ■ autophagy ■ everolimus ■ inflammation

Atherosclerosis is a chronic inflammatory disorder of the arterial wall and represents one of the leading causes of death and disability in many parts of the Western world. Thanks to the development of cholesterol-lowering drugs, the life span and general well-being of patients with coronary artery disease (CAD) have been significantly improved. However, a large group of patients with CAD does not benefit from current lipid-lowering strategies. Anti-inflammatory drugs have been examined, but the results remain contradictory and often disappointing. Therefore, additional therapy for inhibition or regression of atherosclerosis is needed. A growing body of evidence suggests that the mammalian target of rapamycin (mTOR) inhibitor everolimus may be a promising compound for the treatment of CAD. Indeed, in vitro studies indicate that everolimus has pleiotropic antiatherosclerotic effects that may prevent or delay the pathogenesis of atherosclerosis. First, inhibition of mTOR abrogates the activation of the mTOR substrates p70 S6 kinase and initiation factor 4E binding protein 1, elicited by a diversity of mitogenic stimuli, resulting in inhibition of smooth muscle cell (SMC) migration and proliferation. As a consequence, implantation of stents coated with everolimus is already a routine clinical practice to prevent restenosis after percutaneous angioplasty. Second, everolimus has significant antimacrophage activity that is mediated by at least 2 potentially independent mechanisms, namely the inhibition of monocyte chemotaxis and the induction of macrophage death by autophagy. Third, everolimus prevents lipid accumulation in macrophages and SMCs due to inhibition of endogenous cholesterol synthesis, downregulation of low-density lipoprotein (LDL) receptors and scavenger receptors, as well as upregulation of cholesterol efflux genes. Other beneficial effects include upregulation of endothelial nitric oxide synthase (eNOS) in low shear regions, which are susceptible to atherosclerosis, and inhibition of hyaluronic deposition by SMCs, a product that stimulates monocyte adhesion to the extracellular matrix. Studies in different animal models confirm the general notion that everolimus strongly inhibits plaque progression.

Although everolimus has a wide margin of safety and may be suitable for daily oral administration, systemic administration of everolimus at levels suitable for treating atherosclerosis may lead to systemic immunosuppression,

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and such use is not approved. However, stents eluting everolimus or rapamycin (sirolimus) are commonly used in daily practice. Because everolimus is less lipophilic and has a shorter elimination half-life (approximately 30 hours) than rapamycin,20 everolimus-eluting stents are superior, both on safety and efficacy, to rapamycin-eluting stents.21 Still, the potential adverse effects of local everolimus treatment on atherosclerotic plaques have not been systematically analyzed. Given that everolimus-eluting stents deplete macrophages in established plaques through autophagy9,10 and that this process has important modulatory effects on inflammation,22–26 we analyzed in the present study the potential adverse effects associated with everolimus-induced mTOR inhibition by using everolimus-eluting stents and in vitro macrophage cultures.

Methods

Cell Culture
Primary murine macrophage precursors were obtained by differentiating non-adherent macrophage precursors from femoral and tibial bone marrow of C57BL/6 mice for at least 5 days with L-cell-conditioned medium containing macrophage colony-stimulating factor (M-CSF).21 In some experiments, macrophage precursors were isolated from bone marrow of Aeg1/18 mice,20 crossed with macrophage-specific Cre mice (Cre recombinease under control of the lyosome M promoter). Macrophages were then starved in Earle Balanced Salt Solution (EBSS; Invitrogen, Carlsbad, CA) or treated with 10 μmol/L everolimus (Novartis, Basel, Switzerland) in RPMI 1640 medium (Invitrogen). Both EBSS and RPMI were supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and antibiotics (vide supra).

Macrophages were precipitated with 10% trichloroacetic acid, resuspended in 0.2 N NaOH, and measured by liquid scintillation counting. Proteins were precipitated with 10% trichloroacetic acid, resuspended in 0.2 N NaOH, and measured by liquid scintillation counting.

Transmission Electron Microscopy
Macrophages were fixed in 0.1 mol/L sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 hours, then rinsed (3 × 10 minutes) in 0.1 mol/L sodium cacodylate-buffered (pH 7.4) 7.5% saccharose and postfixed in 1% OsO4 solution for 1 hour. After dehydration in an ethanol gradient (70% ethanol for 20 minutes, 96% ethanol for 20 minutes, 100% ethanol for 2 × 20 minutes), samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Tecnai G2 Spirit BioTwin electron microscope (FEI, Hillsboro, OR) at 80 kV.

Western Blotting
Macrophages were lysed in an appropriate volume of Laemmli sample buffer (Bio-Rad, Hercules, CA). Proteins secreted by macrophages were precipitated with 100% trichloroacetic acid before homogenization in Laemmli sample buffer. Samples were then heat-denatured for 5 minutes in boiling water and loaded on an SDS polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Billerica, MA) according to standard procedures. Membranes were blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% nonfat dry milk (Bio-Rad) for 1 hour. After blocking, membranes were probed overnight at 4°C with primary antibodies in antibody dilution buffer (TBS-T containing 1% nonfat dry milk), followed by 1-hour incubation with secondary antibody at room temperature. Antibody detection was accomplished with SuperSignal West Pico or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL), using a Lumi-Imager (Roche).

The following rabbit polyclonal antibodies were used: anti-LC3 from Novus Biologicals (Littleton, CO), anti-Atg7, anti–phospho-p70 S6 kinase (Thr389), anti–eIF2α, anti–phospho-eIF2α (Ser51), anti–p38 MAP kinase, anti–phospho-p38 (Thr180/Tyr182) MAP kinase, anti–ATF2, anti–phospho–ATF2 (Thr71), anti–phospho–cPLA2 (Ser505), anti–NF-kB p65, and anti–phospho–NF-kB p65 (Ser536) from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti–caspase-3 (clone 19) and anti–β-actin (clone AC-15) were from BD Transduction Laboratories (Lexington, KY) and Sigma, respectively. Peroxidase-conjugated secondary antibodies were purchased from Dako (Glostrup, Denmark).

Cytokine Analysis
Cytokines were detected in the culture medium of macrophages, using the Mouse Cytokine Antibody Array 1 from Ray Biotech (Norcross, GA) according to the instructions of the manufacturer. Furthermore, colorimetric sandwich ELISA kits (R&D Systems) were used for quantitative determination of secreted IL-6, MCP1, Rantes, and TNFα.

Stent Implantation
New Zealand White rabbits (Merelbeke, Belgium) were fed a 0.3% cholesterol-supplemented diet for 42 weeks to induce atherosclerotic plaques in the aorta. Subsequently, Multi-Link Vision stents (Abbott Vascular, Santa Clara, CA) coated with everolimus/clobetasol 20:0 g/cm², everolimus/clobetasol 20:1 g/cm², or durable polymer only were deployed in the infrarenal atherosclerotic aorta of each rabbit as previously described.9 Stents were deployed at a distance of minimum 1 cm from each other. Special care was made not to deploy stents over any major bifurcations or side branches. After stent implantation, all rabbits were continued on a 0.3% cholesterol-supplemented diet for 4 weeks to evaluate the response to drug-eluting stents. Animals were observed at least once daily for general health and appearance. Finally, the stented aorta segments were excised, fixed in 4% neutral buffered formalin for 24 hours, and embedded in glycol methacrylate before sectioning. One segment adjacent to each stent was snap-frozen in liquid nitrogen and stored at −80°C for real-time quantitative RT-PCR analysis. Experiments were approved by the local ethics committee.

Immunohistochemical Analysis
Immunohistochemical detection of macrophages and SMCs were carried out by an indirect antibody conjugate technique. Briefly, slides were deplastified and endogenous peroxidase was blocked by 3% H2O2 for 15 minutes. Staining of macrophages required pretreatment of tissue with trypsin/citrate (microwave for 15 minutes...
at 95°C). Slides were then incubated overnight with an anti-rabbit macrophage antibody (clone RAM-11, Dako; 1:1000) or antibody to α-SMC actin (clone, Sigma; 1:1000). After rinsing with PBS, samples were incubated with HRP-conjugated rabbit anti-mouse (DAKO) for 60 minutes to detect the primary antibody. Slides were rinsed in PBS, and 3,3’-diaminobenzidine was used for visualizing the antibody complex. The slides were counterstained with hematoxylin for 4 minutes, dehydrated, and covered with dibutylphthalate.

Real-Time Quantitative RT-PCR
Total RNA was isolated from cultured macrophages or frozen rabbit aorta segments with the Absolutely RNA Microprep Kit (Agilent Technologies, Santa Clara, CA), following the manufacturer’s instructions. Reverse transcription of macrophage RNA was performed at 42°C, using oligo(dT)12-18 and Superscript II Reverse Transcriptase (Invitrogen). TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for IL-6, MCP1, Rantes, and TNF were then performed on an ABI Prism 7300 sequence detector system (Applied Biosystems). The parameters for PCR amplification were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative abundance of TNF mRNA in rabbit aorta was assessed using Taqman gene expression assay Oe03397715_m1 (Applied Biosystems), using the 2-step RTqPCR Core kit (Eurogentec, Fremont, CA). PCR cycling parameters were: reverse transcription at 48°C for 30 minutes, inactivation of RT at 95°C for 10 minutes, followed by 40 cycles consisting of incubations at 95°C for 15 seconds and 60°C for 1 minute. Relative expression of mRNA species was calculated using the comparative Ct (threshold cycle) method. All data were controlled for quantity of RNA input by performing measurements on the endogenous reference gene β-actin (mouse macrophages) or 18SrRNA (rabbit aorta).

Statistical Analysis
Results are expressed as mean±SEM. All analyses were performed using SPSS software (version 18.0). P<0.05 was considered statistically significant. If variances were unequal, logarithmically transformed data were used.

Results
mTOR Inhibition Through Starvation or Everolimus Has Differential Effects on De Novo Protein Synthesis
Mouse bone marrow–derived macrophage precursors were isolated and, after in vitro differentiation into macrophages, either starved or treated with everolimus to inhibit mTOR. Western blot analysis of the phosphorylation status of the downstream mTOR target p70 S6 kinase confirmed rapid mTOR inhibition (within 15 minutes of treatment in both conditions) (Figure 1A). Starvation-induced mTOR inhibition was associated with dephosphorylation of eIF2α (Figure 1B) and enhanced de novo protein synthesis (Figure 1C). In contrast, everolimus-induced mTOR inhibition stimulated eIF2α phosphorylation (Figure 1B) and led to inhibition of de novo protein synthesis, even though this inhibition was partial as compared with cycloheximide (Figure 1C).

Everolimus Stimulates Secretion of Proinflammatory Cytokines by Macrophages
Both starvation and everolimus-induced mTOR inhibition were associated with hyperphosphorylation of p38 MAPK and downstream p38 substrates such as transcription factor ATF2 and cPLA2, though these effects were much more pronounced after treatment with everolimus (Figure 2). Neither starved nor everolimus-treated cells significantly activated transcription factor NF-κB, in contrast to LPS, which was used as control (Figure 2). Because p38 MAPK plays a central role in inflammation,50 the culture medium of starved and everolimus-treated macrophages was screened for the presence of 22 key inflammatory cytokines/chemokines using a cytokine protein array system. Low cytokine levels were found in the culture medium of starved macrophages and untreated control cells (Figure 3A and Figure I in the online-only Data Supplement), even after 3 days of incubation. However, everolimus-treated cells released large amounts of several proinflammatory cytokines and...
Everolimus-Induced Cytokine Secretion Is Independent of Autophagy

Macrophage death was induced after starvation or treatment with everolimus, albeit macrophages died faster when exposed to everolimus (Figure VI in the online-only Data Supplement). Secretion of cytokines, with the exception of MCP1, could be inhibited by the p38 MAPK inhibitor SB202190 (Figure 3C). The glucocorticoid clobetasol blocked secretion of all 4 cytokines (Figure V in the online-only Data Supplement).

Rapamycin Has Similar Effects on Macrophages as Compared With Everolimus

Macrophages exposed to rapamycin revealed similar features as everolimus-treated cells. These features included rapid dephosphorylation of p70 S6 kinase, inhibition of de novo mRNA synthesis of IL-6, MCP-1, Rantes, and TNFα as compared with untreated controls (Figure II in the online-only Data Supplement). To confirm autophagosome formation, cells were examined by means of transmission electron microscopy. Starved and everolimus-treated cells showed an intact nonpyknotic nucleus and numerous autophagic vacuoles in the cytoplasm, whereas control cells did not display vacuolization (Figure VIIB in the online-only Data Supplement).

Because everolimus evokes features of autophagy in macrophages, we examined whether autophagy induction could play a role in everolimus-induced cytokine secretion. For this purpose, macrophage precursors were isolated from mice with a macrophage-specific deletion of the essential autophagy gene Atg7 (Figure 4A). After differentiation, cells were exposed to everolimus. ELISA showed comparable levels of cytokine synthesis after everolimus treatment as compared with wild-type cells (Figure 4B).
protein synthesis, induction of macrophage death, hyperphosphorylation of p38, and stimulation of cytokine production both at the mRNA and protein level (Figure VIII in the online-only Data Supplement).

**Clobetasol Did Not Affect Everolimus-Induced Macrophage Clearance in Rabbit Atherosclerotic Plaques**

Stents eluting everolimus, everolimus/clobetasol, or polymer control stents were implanted in atherosclerotic arteries of cholesterol-fed rabbits. After 1 month, plaques treated with polymer-control stents were characterized by the presence of macrophages and SMCs (Figure 5). In contrast, in plaques treated with everolimus-eluting stents, the macrophage content was significantly reduced, both around the stent struts as well as within or throughout the plaque (Figure 5). Clearance of macrophages by stents coated with both everolimus and clobetasol was as efficient as everolimus-eluting stents (Figure 5). We did not observe a significant reduction in SMC content by everolimus, although there was a trend toward a minimal reduction (Figure 5). To examine the effects of everolimus on TNFα production, RNA was isolated from everolimus-treated plaque segments adjacent to the stented regions and from untreated atherosclerotic control segments. Real-time RT-PCR showed increased (2.1 ± 0.3-fold) TNFα mRNA levels versus control (P < 0.016, 1-way ANOVA, followed by Bonferroni test) (Figure IX in the online-only Data Supplement). Combined treatment with everolimus/clobetasol normalized TNFα mRNA to control levels (Figure IX in the online-only Data Supplement).

**Discussion**

We previously reported that stent-based delivery of the mTOR inhibitor everolimus in atherosclerotic plaques from cholesterol-fed rabbits leads to a marked reduction in macrophage content through selective induction of macrophage death. Because macrophages degrade extracellular collagen in advanced plaques and stimulate SMC loss, stent-based delivery of everolimus may be a promising novel strategy for stabilization of vulnerable atherosclerotic plaques. However, inhibition of mTOR and/or induction of macrophage death might be accompanied by adverse effects. In the present study, we demonstrate that everolimus triggers the release of several proinflammatory cytokines and chemokines such as IL-6, MCP1, Rantes, and TNFα. Similar effects were observed with rapamycin. Yet, cytokines were not released by macrophages undergoing starvation, a condition known to inhibit mTOR similar to everolimus, suggesting that mTOR inhibition is not sufficient to induce cytokine release (Figure 6).
A

B

Figure 5. Depletion of macrophages in rabbit atherosclerotic plaques was not affected by clobetasol. Stents coated with durable polymer, everolimus (evero) or everolimus/clobetasol (evero + CB) were deployed in the infrarenal aorta of cholesterol-fed rabbits. Four weeks after stent implantation, stented arteries were immunohistochemically stained for RAM11 (macrophages) (A) or α-smooth muscle cell actin (B). S indicates stent strut. Scale bar = 200 μm. **P<0.01 versus polymer (1-way ANOVA, followed by Dunnett test, n=8).

Moreover, because sustained starvation leads to macrophage death, a decrease in macrophage viability is not a prerequisite for cytokine synthesis. Interestingly, everolimus-induced mTOR inhibition stimulated eIF2α phosphorylation and led to induction of de novo protein synthesis (Figure 6). These effects were not observed after starvation. On the contrary, starvation of macrophages was associated with dephosphorylation of eIF2α and enhanced de novo protein synthesis. Because protein synthesis inhibitors have the potential to induce selective macrophage death in atherosclerotic plaques,31,32 we assume that inhibition of protein translation by everolimus is a stressful condition for macrophages that could mediate cytokine synthesis. Indeed, everolimus substantially increased the phosphorylation of p38 MAPK, a key regulator of many proinflammatory proteins, and activated downstream p38 targets such as the transcription factor ATF2 and cPLA2. Hyperphosphorylation of p38 MAPK is also observed after treatment of macrophages with protein synthesis inhibitors,33 suggesting that inhibition of de novo protein synthesis after everolimus treatment drives p38 activation (Figure 6). Whether mTOR inhibition is required to downregulate protein translation is presently unclear. mTOR function is indeed essential for efficient translation of specific mRNAs,33 yet this theory is contradictory with enhanced translation after starvation. Possibly, everolimus (or analogs) may also inhibit the translation machinery directly or through unknown intermediate proteins.

Inhibition of p38 MAPK by the p38-specific inhibitor SB202190 blocked secretion of IL-6, Rantes, and TNFα but not MCP1 synthesis, indicating that cytokine production is mainly but not exclusively controlled by p38 MAPK activation. Regulation of MCP1 is relatively complex and may require activation of NF-κB, platelet-derived growth factor, GM-CSF, or cytokines such as IFNγ and IL-1β.34–36 Western blotting showed that NF-κB was not activated by everolimus. To fully suppress everolimus-mediated cytokine secretion, macrophages were incubated with clobetasol, which is one of the most powerful corticosteroids with anti-inflammatory activity.37 Also of note, crosstalk exists between the glucocorticoid receptor (GR) and mTOR. Rapamycin can potentiate mRNA expression of GR target genes by stimulating GR translocation to the nucleus38 and by inhibiting P-glycoprotein, which actively extrudes some steroids from cells.39 This makes corticosteroids ideal compounds to suppress everolimus-induced cytokine secretion. Indeed, clobetasol inhibited the everolimus-induced release of all cytokines studied, including MCP1. The precise mechanism is unclear, although corticosteroids are thought to act by the induction of cPLA2 inhibitory proteins, collectively called lipocortins.40 It is postulated that these proteins control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes by inhibiting the release of their common precursor arachidonic acid. Furthermore, corticosteroids are known to regulate the immune system at the transcriptional and posttranscriptional level, for example, by increasing the rate of IκBα protein synthesis, which in turn traps activated NF-κB.40,41

Figure 6. Schematic overview of signaling pathways in macrophages after mTOR inhibition. Starvation as well as treatment with chemical mTOR inhibitors such as everolimus and rapamycin may lead to inhibition of mTOR and induction of macrophage autophagy. However, in contrast to starvation, everolimus (or rapamycin) has an additional effect, namely inhibition of de novo protein synthesis, that triggers p38 MAPK activation and cytokine synthesis. The latter can be inhibited by the p38-specific inhibitor SB202190 or the glucocorticoid clobetasol.
adjacent to the stented region, yet did not affect the efficiency of everolimus-induced macrophage clearance. Therefore, local administration of clobetasol is an attractive strategy to suppress the proinflammatory responses after everolimus-induced macrophage death. Importantly, everolimus-eluting stents used in this study were coated with 20 μg everolimus per cm², which is 5-fold lower than the dose used in our previous study⁴ or in commercially available Xience V everolimus-eluting coronary stents. Still, macrophage depletion was as efficient as with the standard dose of 100 μg/cm².

Inhibition of mTOR by everolimus caused induction of autophagy, a cellular housekeeping mechanism that is important for the elimination of defective and damaged organelles.⁴² However, sustained stimulation of autophagy results in macrophage death. Macrophages deficient for the essential autophagy, a cellular housekeeping mechanism that is important for the elimination of defective and damaged organelles.⁴²

With regard to translation of the present data into a clinical setting, at least 1 critical issue should be mentioned. Our findings were derived from cell culture experiments and from stent studies in rabbits. Because a 3-year follow-up of the Xience V everolimus-eluting stents in patients revealed improved clinical outcomes and a favorable safety profile,⁴³ it remains to be determined whether everolimus-induced cytokine production by autophagic macrophages after stenting also occurs in men, and if so, has any clinical significance. In addition, rabbit plaques did not show adverse effects (except delayed endothelial healing) after implantation of everolimus-eluting stents, at least not in a short-term follow-up (1 month),⁴⁴ further indicating that the clinical significance of the present data is still to be determined.

In conclusion, our results showed that everolimus- but not starvation-induced mTOR inhibition triggers the release of several proinflammatory cytokines before induction of macrophage autophagic death. Everolimus-induced cytokine secretion is not dependent on the induction of autophagy but most likely results from inhibition of de novo protein synthesis and p38 MAPK activation. These findings provide a rationale for the use of stents eluting both everolimus and an anti-inflammatory agent, such as the glucocorticoid clobetasol that proved to suppress inflammatory responses adequately without affecting the ability of everolimus to deplete macrophages in atherosclerotic plaques.

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Disclosures
None.

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Supplemental material

Everolimus triggers cytokine release by macrophages

Rationale for stents eluting everolimus and a glucocorticoid

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Figure I. Layout of the Mouse Cytokine Antibody Array as used in Figure 3A, supplemental Figure VIII and IX. P=positive control, N=negative control. Please note that IL-12 antibody reacts with both IL-12p40 and IL-12p70. IL12* recognizes only IL-12p70.
Figure II. mRNA expression of IL-6, MCP1, Rantes and TNFα in mouse macrophages after treatment with everolimus. Macrophages were incubated in RPMI medium with or without 10 µM everolimus for 8 hours. After treatment, total RNA was isolated and examined via real time RT-PCR. *P<0.05, **P<0.01 versus control (one-sample test versus test value 1, n=10).
Figure III. Everolimus exacerbates secretion of TNFα and MCP1 in response to LPS treatment of mouse macrophages. Cells were incubated in RPMI medium (-) or in RPMI supplemented with 10 µM everolimus (Ev), LPS (0.1 to 100 ng/ml) or a combination of LPS (0.1-100 ng/ml) and everolimus (10 µM) for 24 hours. Secretion of TNFα (A) and MCP1 (B) was analyzed via ELISA. **P<0.01, ***P<0.001 versus control (-); *P<0.05, **P<0.01 versus LPS (unpaired student’s t test, n=5).
Figure IV. Everolimus treatment of human macrophages stimulates secretion of the pro-inflammatory cytokine IL-6. (A) Human macrophages were incubated in RPMI (control) or RPMI supplemented with 10 µM everolimus for 8 hours. Thereafter, IL-6 mRNA was analyzed by real-time RT-PCR. (B) Human macrophages were incubated in RPMI (control) or RPMI supplemented with 10 µM everolimus for 24 hours. The culture medium was then analyzed for the presence of IL-6 via ELISA. *P<0.05, ***P<0.001 versus control (unpaired student’s t test, n=6-8).
Figure V. Everolimus-induced cytokine secretion is inhibited by the corticosteroid clobetasol. Mouse macrophages were incubated in RPMI medium (control) or RPMI supplemented with 10 µM everolimus and clobetasol (CB, 0-1 µM) for 24 hours. Secretion of IL-6, MCP1, Rantes and TNFα was analyzed by ELISA. ***P<0.001 versus all other groups (one-way ANOVA, followed by Bonferroni test, n=5).
**Figure VI.** Viability of mouse macrophages after starvation in Earle’s Balanced Salt Solution or treatment with 10 µM everolimus in RPMI medium for 0-72 hours or 0-48 hours, respectively. *P*<0.05, ***P*<0.001 versus 0 hours (one-way ANOVA, followed by Dunnett test, n=4).
Figure VII. Characterization of mouse macrophage death induced by starvation or everolimus. A, Macrophages were incubated in Earle’s Balanced Salt solution or treated with 10 µM everolimus for 0-48 hours. To characterize the type of cell death induced, cleavage of procaspase-3 (procasp-3) and DNA fragmentation (both apoptosis markers) were analyzed using western blotting and flow cytometry, respectively. B, Macrophages were starved or everolimus treated for 0-60 minutes. Western blot analysis of LC3 processing was performed to detect autophagy. Ultrastructural features of untreated macrophages (control) as well as macrophages starved or treated with 10 µM everolimus for 24 hours were examined via transmission electron microscopy. Both starvation and everolimus treatment induced formation of autophagic vesicles (arrows). N indicates nucleus. Scale bar=2 µm.
Figure VIII. Macrophages that were exposed to rapamycin revealed similar features as everolimus-treated cells. (A) Mouse macrophages were incubated in serum-containing RPMI medium (control) or RPMI supplemented with 10 µM rapamycin for 0-60 minutes. Phospho-p70 S6 kinase (P-p70S6K, Thr389) was analyzed by western blotting. (B) Macrophages were incubated in serum-containing RPMI (control) or RPMI supplemented with either 10 µM rapamycin or 10 µg/ml cycloheximide (CHX) for 2 hours in the presence of L-35S-methionine/cysteine. 35S-labeled proteins were measured with scintillation counting. ***P<0.001 versus control (one-way ANOVA, followed by Dunnett test, n=5). (C) Macrophage viability after treatment with 10 µM rapamycin in RPMI medium for 0-48 hours. *P<0.05, ***P<0.001 versus 0 hours (one-way ANOVA, followed by Dunnett test, n=5). (D) Western blot analysis of the phosphorylation status of p38 MAPK (Thr180/Tyr182) after incubation of macrophages in RPMI medium containing 10 µM rapamycin for up to 8 hours. (E) Macrophages were incubated in RPMI (control) or RPMI supplemented with 10 µM rapamycin for 24 hours. The culture medium was then analyzed for the presence of 22 different cytokines/chemokines using a cytokine antibody array. The layout of the array is shown in Figure 2. (F) mRNA expression of IL-6, MCP1, Rantes and TNFα in mouse macrophages after treatment with rapamycin. Macrophages were incubated in RPMI medium with or without 10 µM rapamycin for 8 hours. After treatment, total RNA was isolated and examined via real time RT-PCR. **P<0.01, ***P<0.001 versus control (one-sample test versus test value 1, n=10).
**Figure IX.** Combined local treatment of rabbit atherosclerotic plaques with everolimus and clobetasol (CB) inhibits everolimus-induced TNFα mRNA synthesis. RNA was isolated from everolimus-treated or everolimus/clobetasol-treated plaque segments adjacent to the stented regions and from untreated atherosclerotic control segments. After reverse transcription of RNA into cDNA, TNFα mRNA levels were evaluated by real-time PCR. **P<0.01 versus control (Mann-Whitney test, n=3-6).**