3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase–Independent Inhibition of CD40 Expression by Atorvastatin in Human Endothelial Cells

Andreas H. Wagner, Matthias Gebauer, Björn Güldenzoph, Markus Hecker

Objective—3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) exert potent anti-inflammatory effects that are independent of their cholesterol-lowering action. We have investigated the effects of these drugs on cytokine-stimulated CD40 expression in human cultured endothelial cells and monocytes.

Methods and Results—Reverse transcription–polymerase chain reaction and Western blot analysis revealed that treatment of either cell type with atorvastatin, cerivastatin, or pravastatin (1 to 10 μmol/L) inhibited interferon-γ plus tumor necrosis factor-α–stimulated CD40 expression by ~50%, an effect that was not reversed by the HMG-CoA reductase product mevalonic acid (400 μmol/L). In contrast, mevalonic acid prevented the inhibitory effect of atorvastatin on cytokine-stimulated vascular cell adhesion molecule-1 expression and subsequent adhesion of THP-1 monocytes to the cultured endothelial cells. Transcription factor analysis revealed an inhibition by atorvastatin of nuclear factor-κB plus signal transducer and activator of transcription-1–dependent de novo synthesis of interferon regulatory factor-1, governing cytokine-stimulated CD40 expression in these cells. One consequence of this statin-dependent downregulation of CD40 expression was a decrease in CD40 ligand–induced endothelial interleukin-12 expression.

Conclusions—By interfering with cytokine-stimulated CD40 expression in vascular cells, statins thus seem capable of attenuating CD40 ligand–induced proinflammatory responses, including atherosclerosis. In addition, they point to the coexistence of HMG-CoA reductase–dependent and –independent effects of statins in the same cell type. (Arterioscler Thromb Vasc Biol. 2002;22:1784-1789.)

Key Words: 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors • endothelial cells • atherosclerosis • CD40 • interleukin-12

A cell surface molecule, CD40 belongs to the family of tumor necrosis factor (TNF) receptors predominantly expressed by antigen-presenting cells but also by a variety of nonimmune cells.1 In endothelial cells, CD40 stimulation via the corresponding ligand (CD40L) causes an increased expression of adhesion molecules and chemokines that promotes homing and extravasation of leukocytes at sites of inflammation.2,3 Moreover, in response to CD40 stimulation, endothelial cells are capable of producing bioactive interleukin (IL)-12,4 a potent differentiation factor for naive T helper cells that stimulates their clonal expansion into Th1 cells.5 As a consequence, attenuation of CD40 expression and/or activity in endothelial cells appears to be a promising target for interfering with acute and chronic inflammatory responses, including atherosclerosis.

Constitutive expression of CD40 in human endothelial cells is markedly enhanced on exposure to certain proinflammatory cytokines, namely, the combination of interferon (IFN)-γ with TNF-α.6 Uregulation of CD40 expression under these conditions is mediated primarily by nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)-1–dependent de novo synthesis of the transcription factor interferon regulatory factor (IRF)-1.6

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, collectively referred to as statins, exert therapeutic effects beyond lowering plasma cholesterol levels (see review7). Thus, statins improve endothelial function through the upregulation of NO synthase expression,8 they attenuate superoxide formation in these cells,9 and they inhibit proinflammatory cytokine formation in leucocytes and adhesion molecule expression in endothelial cells (see review10). One explanation for these effects is that metabolites of the HMG-CoA reductase product mevalonic acid are required for the posttranslational modification and, hence, the functional activity of small GTP-binding proteins (G proteins), which play an important role in the cellular processes mentioned above.11

In the present study, we have investigated the hypothesis that statins are capable of interfering with cytokine-induced CD40 expression in human endothelial cells and monocytes...
and, as a consequence, with the subsequent changes in gene expression hence cell function triggered by CD40L.

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords as described previously and cultured in medium 199 containing 20% FBS (Life Technologies), 50 U/mL penicillin, 50 μg/mL streptomycin, 10 U/mL nystatin, 5 mmol/L HEPES, 5 mmol/L TES, 1 μg/mL heparin (Sigma-Aldrich), and 40 μg/mL endothelial cell growth factor (PromoCell). As demonstrated previously, the cultured HUVECs were not contaminated with CD45+ or CD14+ leukocytes.

The human premonocytic cell line THP-1 and the CD40L-positive T-cell leukemia line Jurkat D1.1 (both purchased from the American Type Culture Collection) were cultured in RPMI 1640 medium (Life Technologies) containing 10% FBS and antibiotics as described previously. For coinoculation experiments (12 hours), the Jurkat cells were added to the cultured HUVECs at 1×10^6 cells per well. The CD40L-neutralizing antibody (clone TRAP-1, BD Biosciences), was used at a concentration of 40 μg/mL. Flow cytometry of Jurkat cells with a mouse anti-human CD40L monoclonal primary antibody (DAKO) and a FITC-labeled polyclonal goat anti-mouse secondary antibody (BD Biosciences) was performed as described previously.

Native human blood monocytes were kindly provided by Dr. Afsaneh Sorouri, Department of Immunology, University of Goettingen, and prepared as follows: Leukocytes from blood of healthy donors were collected by leukopheresis, and peripheral blood mononuclear cells were separated by density gradient centrifugation on a Ficoll-Hypaque discontinuous gradient. They were cultured at a density of 1×10^6 cells per well. The CD40L-neutralizing antibody (clone TRAP-1, BD Biosciences) was used at a concentration of 40 μg/mL. Flow cytometry of Jurkat cells with a mouse anti-human CD40L monoclonal primary antibody (DAKO) and a FITC-labeled polyclonal goat anti-mouse secondary antibody (BD Biosciences) was performed as described previously.

Preparation of nuclear extracts from the cultured cells and subsequent nondenaturing 4% polyacrylamide gel electrophoresis were carried out as described previously. The double-stranded gel-shift oligonucleotides (Santa Cruz Biotechnology) for STAT-1, NF-κB, and IRF-1 were end-labeled with [γ-^32P]ATP by using the 5’-end-labeling kit from Amersham Pharmacia Biotech. The specificity of the binding reaction was monitored by performing the assay in parallel with the same samples in the presence of a 100- to 1000-fold excess of the nonlabeled oligonucleotide. For supershift analyses, the appropriate gel shift antibody (Santa Cruz Biotechnology) and nuclear extracts were preincubated at ambient temperature for 60 minutes before the electrophoretic mobility shift analysis (EMSA) was performed.

**Electrophoretic Mobility Shift Analysis**

Preparation of nuclear extracts from the cultured cells and subsequent nondenaturing 4% polyacrylamide gel electrophoresis were carried out as described previously. The double-stranded gel-shift oligonucleotides (Santa Cruz Biotechnology) for STAT-1, NF-κB, and IRF-1 were end-labeled with [γ-^32P]ATP by using the 5’-end-labeling kit from Amersham Pharmacia Biotech. The specificity of the binding reaction was monitored by performing the assay in parallel with the same samples in the presence of a 100- to 1000-fold excess of the nonlabeled oligonucleotide. For supershift analyses, the appropriate gel shift antibody (Santa Cruz Biotechnology) and nuclear extracts were preincubated at ambient temperature for 60 minutes before the electrophoretic mobility shift analysis (EMSA) was performed.

**Endothelial Cell–Leukocyte Interaction**

Confluent HUVEC monolayers grown on glass slides were washed with HEPES-Tyrode solution (composition in mmol/L: NaCl 137, KCl 2.7, CaCl 2, MgCl 2 0.25, NaHPO 4 0.4, sodium HEPES 10, and d-glucose 5) containing 1.5% polynvinylpyrrolidone (PVP, Sigma-Aldrich) and mounted on the bottom of a parallel-plate flow chamber with a height of 2.5 mm and a volume of 260 μL (Warner Instruments) that was placed on the stage of an Axiovert S100 TV microscope (Zeiss). A heated platform and an in-line solution heater (Warner Instruments) were used to maintain the cells at 37°C. The cultured cells were superfused at a defined wall shear stress of 5 dynes/cm² and a shear rate of 10 s⁻¹ by using a precision pump (Ismatec). Cell–cell interaction was monitored at ×20 magnification with a SPOT RT color CCD camera (Diagnostic Instruments). The HUVEC monolayer was first superfused for 10 minutes with HEPES-Tyrode/PVP, followed by single-passage superfusion with 1.5×10⁶ THP-1 cells (5×10⁵ THP-1 cells/mL) in HEPES-Tyrode/PVP for 10 minutes. Thereafter, the flow chamber was rinsed with HEPES-Tyrode/PVP, and 3 images from different regions were recorded for subsequent automatic counting of adherent cells by using the MetaMorph V3.0 software package (Universal Imaging).

**Data Analysis**

Unless indicated otherwise, results are expressed as mean±SEM of n observations with different batches of cells. One-way ANOVA followed by a Dunnett multiple comparisons test was used to determine differences between the means and the corresponding control value with P<0.05 considered statistically significant.
Results

Effects of HMG-CoA Reductase Inhibitors on CD40 Expression

Treatment of the cultured endothelial cells with 10 μmol/L atorvastatin, 10 μmol/L pravastatin, or 1 μmol/L cerivastatin resulted in a decrease in TNF-α (100 U/mL) plus IFN-γ (100 U/mL)–stimulated CD40 mRNA expression by 41±7%, 45±10%, and 38±11%, respectively (n=4, refer to Figure 1a; for an exemplary RT-PCR analysis, see Figure I in the online data supplement, which can be accessed at http://atvb.ahajournals.org). Time-course studies (1- to 20-hour preincubation) with atorvastatin (10 μmol/L) revealed that a preincubation period of 1 hour (with the statin remaining in the medium for the rest of the experiment) was sufficient to yield this effect, whereas it was no longer effective when added 2 hours after cytokine exposure (Figure 1b). It is important to note that neither statin at the concentration used exerted any cytotoxic effect, as judged by the MTT assay (not shown).

Interestingly, the inhibitory effect of atorvastatin on CD40 mRNA expression was not altered by mevalonic acid (400 μmol/L). Sufficient cellular uptake of the HMG-CoA reductase product under the chosen experimental conditions (coincubation with atorvastatin) was confirmed by monitoring the intracellular accumulation of [14C]mevalonic acid over time (Figure 2b). Moreover, effective circumvention of atorvastatin blockade of HMG-CoA reductase was also demonstrated by reversal of the inhibitory effect of atorvastatin on cytokine-stimulated VCAM-1 expression (Figure 2c).

In addition to CD40 mRNA expression, pretreatment of the endothelial cells with atorvastatin also inhibited TNF-α plus IFN-γ–stimulated CD40 protein expression in a concentration-dependent manner (Figure 3a; see also Figure IIa in the online data supplement, which can be accessed at http://atvb.ahajournals.org). This effect was insensitive to mevalonic acid too (Figure 3a). Moreover, neither farnesyl pyrophosphate nor geranylgeranyl pyrophosphate (both at 10 μmol/L) reversed the inhibitory effect of atorvastatin on cytokine-stimulated CD40 protein expression (please see Figure IIa in the online data supplement, which can be accessed at http://atvb.ahajournals.org). The observed HMG-CoA reductase–independent effect of atorvastatin was further supported by the fact that inhibitors of farnesyl transferase or geranylgeranyl transferase also did not affect cytokine-stimulated CD40 mRNA expression (24-hour pretreatment...
with 10 μmol/L FTI-277 or GGTL-298; please see online Figure 1b) or protein expression (not shown). Furthermore, in the mononuclear cell line THP-1 (Figure 3b) and in freshly isolated human monocytes (Figure 3c), atorvastatin/statin exerted a practically identical effect on cytokine-stimulated CD40 protein expression; i.e., it was concentration dependent (refer to online Figure IIIb and IIIc) and insensitive to mevalonic acid.

Effects of Atorvastatin on Transcription Factor Activation

The aforementioned findings suggested that statins, namely, atorvastatin, inhibit CD40 expression in human vascular cells in response to IFN-γ plus TNF-α stimulation. Therefore, nuclear translocation of transcription factors IRF-1, STAT-1, and NF-κB, which are typically activated by these cytokines and have been implicated in the control of CD40 gene expression in these cells (please refer to Wagner et al6), were examined next. EMSA revealed that in the cultured endothelial cells, 3 hours of exposure to TNF-α plus IFN-γ stimulated nuclear translocation of all 3 transcription factors and that this was attenuated in atorvastatin-treated cells (10 μmol/L, 1-hour preincubation) to a comparable degree (Figure 4). Moreover, the inhibitory effect of atorvastatin on cytokine-stimulated translocation of IRF-1 and NF-κB to the nucleus was not reversed by mevalonic acid (Figure 4).

Specificity of the EMSA (eg, for IRF-1) was confirmed by supershift analyses (please refer to Figure IV in the online data supplement, which can be accessed at http://atvb.ahajournals.org).

Effects of Atorvastatin on Endothelial Cell–Leukocyte Interaction and CD40/CD40L-Mediated IL-12p40 Expression

Finally, to assess the functional relevance of atorvastatin-mediated inhibition of cytokine-stimulated proinflammatory gene expression, we used a parallel-plate flow chamber mimicking physiological flow conditions to monitor endothelial cell–leukocyte interaction. Treatment of the cultured endothelial cells with atorvastatin (10 μmol/L, 1-hour preincubation) markedly reduced TNF-α plus IFN-γ-stimulated THP-1 cell adhesion monitored 12 hours later (Figure 5a). Similar to the inhibition by atorvastatin of cytokine-stimulated VCAM-1 expression, this effect was fully reversed by coincubation with mevalonic acid.

On the other hand, atorvastatin attenuation of cytokine-stimulated CD40 expression significantly inhibited the subsequent CD40L-mediated induction of IL-12p40 mRNA expression, and this effect was mevalonic acid insensitive (Figure 5b). In human endothelial cells, IL-12p40 expression is mediated exclusively through the activation of CD40 (please see Lienenl6), and in line with this finding,

Figure 4. Effects of atorvastatin on transcription factor activation and lack of effect of exogenous mevalonic acid. a, Effects of 1-hour preincubation with 10 μmol/L atorvastatin on the nuclear translocation of STAT-1 and NF-κB (specific DNA-protein complexes are designated by arrows) in the cultured HUVECs stimulated for 3 hours with T/I. Typical EMSA is shown; qualitatively identical results were obtained for each transcription factor with at least 3 further batches of HUVECs. b, Statistical summary (n=3 or 4, with typical EMSA at the bottom) of cytokine-stimulated nuclear translocation of IRF-1 in atorvastatin-treated HUVECs and lack of effect of cotreatment with 400 μmol/L mevalonic acid. *P<0.05 vs T/I. The identity of IRF-1 binding to the labeled oligonucleotides was confirmed by supershift analysis with an appropriate antibody (please see online Figure IV).

Figure 5. Atorvastatin inhibition of cytokine-stimulated endothelial cell–leukocyte interaction and CD40L-induced IL-12p40 expression. a, Statistical summary demonstrating the inhibitory effect of atorvastatin (10 μmol/L, 1-hour preincubation) followed by 12 hours of exposure to T/I on THP-1 cell adhesion to the cultured HUVECs (n=4). *P<0.05 vs control; †P<0.05 vs T/I; and ‡P<0.05 vs T/I+atorvastatin. Coincubation with 400 μmol/L mevalonic acid reversed the inhibitory effect of atorvastatin. b, Statistical summary of the inhibitory effect of atorvastatin (10 μmol/L, 1-hour preincubation followed by 12 hours of exposure to T/I and lack of effect of 400 μmol/L mevalonic acid on CD40L-induced (1×10⁶ Jurkat cells per well) IL-12p40 mRNA expression over 12 hours (n=3 to 5). *P<0.05 vs T/I+CD40L. Note that atorvastatin had no effect on CD40L-induced IL-12p40 expression in cells expressing basal levels of CD40 protein.
endothelial cell IL-12p40 mRNA expression induced by the CD40L-expressing Jurkat cells used for the experiments shown in Figure 5b was virtually abrogated in the presence of a CD40L-neutralizing antibody (please refer to Figure V in the online data supplement, which can be accessed at http://atvb.ahajournals.org).

Discussion

The present findings demonstrate that statins, namely, atorvastatin, reduce the cytokine-induced upregulation of CD40 expression in human endothelial cells and monocytes by interfering with the de novo synthesis of the transcription factor IRF-1. This effect occurs independently of the blockade of HMG-CoA reductase and results in an attenuation of CD40-mediated gene expression, exemplified by the Th1 cytokine IL-12.

Statins mainly prescribed because of their lipid-lowering activity are potent inhibitors of HMG-CoA reductase. Emerging evidence from clinical trials and basic research suggests that this class of drugs has anti-inflammatory properties that may add to their clinical efficacy, eg, by decreasing adhesion molecule expression in patients with established atherosclerosis. Monocyte recruitment into the vessel wall after exposure to proinflammatory cytokines may also be affected by statins because they are capable of inhibiting the expression of monocyte chemoattractant protein-1 and VCAM-1 (the present study) in human endothelial cells.

There are multiple lines of evidence supporting the view of atherosclerosis as a chronic inflammatory disease involving certain components of the immune system. Recently, enhanced expression of CD40 and CD40L has been reported in experimental and human atherosclerotic lesions (see review), and patients with moderate hypercholesterolemia reveal an upregulated CD40/CD40L system that may contribute to the known pro-inflammatory/proatherogenic milieu found in these patients. Thus, short-term administration of statins in these patients significantly decreased CD40 expression in circulating monocytes as well as the serum levels of monocyte chemoattractant protein-1.

We have previously demonstrated that IFN-γ and TNF-α synergistically upregulate CD40 expression in human endothelial cells and in THP-1 monocytes and that this effect is brought about mainly by the NF-κB/STAT-1-dependent de novo synthesis of the transcription factor IRF-1. Although statin inhibition of endothelial cell–monocyte interaction by downregulating monocyte CD11a/CD18/VLA4 expression has been described, our findings support the notion that in addition, statins influence the interaction of these cells by attenuating IFN-γ-dependent IRF-1–mediated gene expression in the endothelial cells. In this respect, 1 μmol/L atorvastatin, a concentration that is ~15-fold higher than the plasma level of atorvastatin in patients receiving the maximum dose of 80 mg/d, was already maximally effective. However, it should be kept in mind that because of its lipophilicity, the statin is likely to accumulate in endothelial cells through passive diffusion, thus exceeding the nominal concentration in the plasma. It is also important to note that in the present study, all statins were dissolved in dimethyl sulfoxide to overcome their different membrane-permeating properties.

In addition to endothelial cell–monocyte interaction, the attenuation of endothelial and monocyte CD40 expression suggests that statins are also capable of interfering with T helper cell–dependent immune responses. This idea is supported by findings that statins inhibit IFN-γ-dependent class II major histocompatibility complex II expression in human endothelial cells. Moreover, in these cells, CD40 stimulation exclusively induces de novo expression of IL-12, which in turn may promote the differentiation and clonal expansion of naive T helper cells into Th1 cells, hence fueling a proinflammatory immune response. Our data show that by downregulating cytokine-stimulated CD40 expression, atorvastatin attenuates the subsequent CD40L-induced expression of IL-12p40, which represents the rate-limiting step in the synthesis of biologically active IL-12 in human endothelial cells. On the other hand, atorvastatin did not seem to affect CD40L expression in the Jurkat T cells, a cell line well established in immunological studies, suggesting that it affects CD40/CD40L-mediated endothelial cell–T-cell interaction primarily through the inhibition of CD40 expression.

Most notably, however, the inhibitory effect of atorvastatin on transcription factor activation and subsequent expression of CD40 was neither reversed by exogenous mevalonic acid, farnesyl pyrophosphate, or geranylgeranyl pyrophosphate nor mimicked by a farnesyl transferase or geranylgeranyl transferase inhibitor. In contrast, mevalonic acid completely reversed the inhibitory effect of atorvastatin on cytokine-stimulated VCAM-1 expression, and in line with this finding (ie, that VCAM-1 is an endothelial cell integrin predominantly involved in leukocyte trafficking and extravasation), it restored the attenuated adhesion of THP-1 monocytes to atorvastatin-treated endothelial cells. Thus, statins seem to affect the expression of different target genes in the same cell type under identical experimental conditions by 2 different mechanisms: one that is HMG-CoA reductase and, presumably, small G-protein dependent and another that occurs independently of the blockade of HMG-CoA reductase.

The mechanism underlying the mevalonoid acid–insensitive modulation of gene expression by statins is as yet unclear. Recently, these drugs have been reported to selectively interact with leukocyte function antigen (LFA)-1, a β2 integrin, via binding to a novel allosteric site within LFA-1. As a consequence, the inflammatory response to thiglycollate in a murine model of peritonitis was suppressed, and this effect was unrelated to the inhibition of HMG-CoA reductase. Perhaps the inhibitory effect of the statins on cytokine-induced CD40 expression in human vascular cells is likewise mediated by their binding to such an integrin-like receptor. In this context, it may be of interest that integrin-dependent modulation of gene expression through LFA-1, increasing the transactivation of an activator protein-1 reporter gene construct in Jurkat T cells, has recently been reported.
of statins in inflammation, including atherosclerosis. Although it is not known whether all statins exert a clinically relevant anti-inflammatory effect or whether one agent is more potent than the other in this regard, our data suggest that the HMG-CoA reductase–independent modulation of gene expression constitutes a class effect too.20,27

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Data supplement

**Figure I:** Effect of 1 h pre-treatment with 10 µM atorvastatin (atorva), 1 µM cerivastatin (ceriva) or 10 µM pravastatin (prava) on CD40 mRNA expression in human cultured endothelial cells after 9 h exposure to TNFα (100 U/ml) plus IFNγ (1000 U/ml; T/I). Typical RT-PCR analysis, amplification of EF-1 cDNA (house-keeping gene) served as an internal standard.

**Figure II:** (a) Effect of atorvastatin on cytokine-stimulated CD40 protein expression and lack of effect of exogenous mevalonic acid (400 µM, meval), farnesylpyrophosphate (10 µM, FPP) and geranylgeranylpyrophosphate (10 µM, GGPP). Human cultured endothelial cells were pre-incubated for 1 h with 10 µM atorvastatin (atorva) in the presence of mevalonic acid, FPP or GGPP and subsequently stimulated for 12 h with 100 U/ml TNFα plus 1000 U/ml IFNγ (n=3; *P<0.05 vs. T/I). (b) Lack of effect of the farnesyltransferase (FT) inhibitor FTI-277 and the geranylgeranyltransferase (GGT) inhibitor GGTI-298 on cytokine-stimulated CD40 mRNA expression. Human cultured endothelial cells were pre-incubated for 24 h with 10 µM FTI-277 or GGTI-298 and subsequently stimulated for 9 h with 100 U/ml TNFα plus 1000 U/ml IFNγ (n=4 with 3 different batches of endothelial cells).

**Figure III:** Concentration-dependent inhibition of CD40 protein expression by atorvastatin in (a) human cultured endothelial cells (EC), (b) THP-1 cells (THP-1) and (c) freshly isolated human monocytes (MØ), and lack of effect of mevalonic acid (400 µM). Representative Western blot analyses with cells pre-treated with atorvastatin (0.1, 1 and 10 µM, as indicated) for 1 h followed by exposure to TNFα (100 U/ml) plus IFNγ (1000 U/ml) for 12 h. Loading and transfer of equal amounts of protein in each lane was verified by reprobing the membrane with an anti-β-actin antibody.

**Figure IV:** Effect of atorvastatin (1 h pre-incubation, 10 µM) on nuclear translocation of IRF-1 and supershift analysis with an anti-IRF-1 immunoglobulin (IgG). The specific complex for IRF-1 and the supershift are designated by arrows. Human cultured endothelial cells were stimulated
for 3 h with TNFα (100 U/ml) plus IFNγ (1000 U/ml). Typical electrophoretic mobility shift analysis.

**Figure V**: Specificity of CD40L-induced IL-12p40 expression in human cultured endothelial cells. The figure demonstrates that the Jurkat cell (1x10⁶ cells/well, 12 h exposure) induced increase in endothelial cell IL-12p40 mRNA expression was abrogated in the presence of a CD40L-neutralizing antibody (40 µg/ml TRAP-1)¹. Typical RT-PCR analysis.

**References**

Wagner et al., Data Supplement Figure I
Wagner et al., Data Supplement Figure II
Wagner et al., Data Supplement Figure III
Wagner et al., Data Supplement Figure IV
Wagner et al., Data Supplement Figure V