Inhibition of B7-1 (CD80) by RhuDex[®] reduces lipopolysaccharide-mediated inflammation in human atherosclerotic lesions

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Background: Atherosclerosis is based on a chronic inflammatory process including the innate and adaptive immune response. Costimulatory molecules and their receptors provide decisive signals for antigen-specific cell activation. The contribution of B7-related pathways to atherosclerosis has hardly been explored.

Methods: In the present study, we investigated the contribution of B7-1 to inflammation and tissue injury in the human plaque microenvironment in order to identify possible target structures of future therapeutic agents ex vivo and in vitro.

Results: Carotid artery plaque stimulation with lipopolysaccharides (LPS) could be significantly inhibited by RhuDex[®], a specific inhibitor of the costimulatory molecule B7-1 ex vivo ($P < 0.001$). Coculture of antigen-presenting cells with T-cells demonstrated that the inhibitory effects of RhuDex[®] derived from reduced T-cell activation. In addition, incubation of monocytes/macrophages with LPS and RhuDex[®] resulted in an inhibitory negative feedback on antigen-presenting cells. Signaling pathways affected by RhuDex[®] seem to be nuclear transcription factor kappa B, activator protein-1, and extracellular signal-regulated kinase 1/2.

Conclusion: The present data support B7-1 alone as an important costimulatory molecule in the context of LPS-mediated inflammation in atherosclerotic lesions. Due to its marked inhibitory effects, RhuDex[®] may be a useful therapy to modulate the inflammatory milieu in atherosclerosis.

Keywords: B7, CD86, costimulation, atherosclerosis

Introduction

The development, progression, and vulnerability of an atherosclerotic lesion results from a chronic inflammatory process.¹⁻³ Various immune cells such as T-cells, macrophages, and dendritic cells (DCs), with their important cytokines tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), interleukin (IL)-6, and chemokines such as monocyte chemoattractant protein-1 (MCP-1), are primarily involved in this process.^{2,4} Following the uptake of autoantigens such as oxidized low density lipoprotein, antigen-presenting cells (APCs) become activated and present the processed antigen predominantly to naïve T-cells.⁵

The costimulatory molecules B7-1/B7-2 (CD80/CD86) on the surface of APCs bind to CD28/cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on T-cells. Interaction between B7-1/B7-2 and the stimulatory receptor CD28 on T-cells is crucial for T-cell activation and proliferation, whereas binding to CTLA-4 resulted in a T-cell activation downregulation.⁶ It is not clear whether B7-1 and B7-2 effects differ significantly from each other. B7-1, which is not constitutively expressed on the surface of APCs,

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is inducible by cell activation. By contrast, B7-2 expression is found on most unstimulated APCs but is further upregulated after stimulation.^{7,8} Recent publications indicated that B7-1 had an inhibitory role in the regulation of T-cell activation while B7-2 showed stimulatory effects.^{9–11} In contrast, other studies suggested that B7-1 and B7-2 could provide similar costimulatory signals to T-cell activation, cytokine production, and generation of cytotoxic T lymphocyte.¹²

The contribution of B7-1/B7-2:CD28/CTLA-4 pathways to atherosclerosis remains controversial.^{4,13–15} In mice, the data for B7-1/B7-2:CD28/CTLA-4 in the context of atherosclerosis showed both a proinflammatory as well as an anti-inflammatory effect.^{13–15} In human lesions, de Boer et al demonstrated that the expression of B7-1 and B7-2 was most intense on macrophages in the superficial layers of the intima.¹⁶ We previously reported that B7-2 was expressed markedly higher in carotid artery plaques of patients with ischemic symptoms such as stroke or transient ischemic attack (TIA) compared to asymptomatic patients.⁴

The current study intended to further investigate the role of B7-1 in the context of a lipopolysaccharide (LPS)-dependent immune response in atherosclerotic lesions by blocking B7-1 with RhuDex®, a small molecule that specifically binds to B7-1.¹⁷

Materials and methods

Ex vivo experiments

Fresh carotid artery plaques were obtained from 12 patients undergoing endarterectomy: six had ischemic symptoms while six were asymptomatic. The current study only used lipid-rich or complicated lesions. Plaque morphology was categorized on the basis of the American Heart Association classification.¹⁸ A lipid-rich lesion was defined as a confluent extracellular lipid core formed with adaptive intimal thickening (type IV lesion, n=6). The presence of a surface defect, thrombosis, and hematoma constituted complicated plaques (type VI lesion, n=6). Approval for this study was given by the Institutional Review Board of the University of Heidelberg, Germany, and appropriate informed consent was obtained from all patients. Specimens were cut into small pieces (3 mm) and randomly planted into wells of a 48-well plate with Roswell Park Memorial Institute (RPMI) medium (with 10% fetal calf serum, 100 U/mL penicillin G, and 100 g/mL streptomycin). Tissues were stimulated with 1 µg/mL of LPS (from *Escherichia coli* 055:B5; Sigma-Aldrich, St Louis, MO, USA), and partly treated with 3 µg/mL RhuDex® (Medigene AG, Planegg/Martinsried, Germany) for

3 hours and 8 hours. Unstimulated cultured plaque pieces served as controls. Cultured tissues were maintained at 37°C in humidified air containing 5% CO₂. After stimulation, plaque tissues were shock-frozen in liquid nitrogen for quantitative polymerase chain reaction analysis (qPCR) or lysed for western blotting. The supernatant was collected and stored at –20°C for enzyme-linked immunosorbent assay (ELISA) protein analysis. The study was conducted according to good clinical practice and in compliance with the 2008 Declaration of Helsinki.

In vitro experiments

Peripheral blood mononuclear cells (PBMCs) were derived from healthy donors by density gradient centrifugation using Ficoll-H (Linaris, Wertheim, Germany). Monocytes were isolated by adherence method as described previously.¹⁹ Briefly, isolated PBMCs were planted in a 6-well plate (Corning Incorporated, Corning, NY, USA) at a density of 20×10⁶/cm² for 20–30 minutes. Afterwards, nonattached cells were removed by vigorously washing three times with 1× phosphate buffered saline. Adherent monocytes were recovered by mechanical detachment. For macrophage differentiation, monocytes were seeded in a 24-well plate in macrophage serum-free medium (Gibco macrophage-SFM; Life Technologies, Carlsbad, CA, USA) with 1% Nutridoma (Roche, Basel Switzerland) and 100 ng/mL recombinant human macrophage-colony stimulating factor (PeproTech, Rocky Hill, NJ, USA) for 6 days. Medium was changed every 2 days with the addition of macrophage-colony stimulating factor. CD4⁺ T-cells were isolated from PBMCs using a Dynabeads Flow Comp™ Human CD4⁺ kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. For coculture, T-cells and monocytes were derived from the same donor.

Monocytes and monocyte-derived macrophages were stimulated with 1 µg/mL LPS and/or 3 µg/mL RhuDex® alone or in combination for indicated periods. Unstimulated cells served as controls. Cells were harvested after incubation for RNA isolation and qPCR analysis. In addition, the supernatant were stored at –20°C until use.

Electrophoretic mobility shift assay

Nuclear proteins from monocytes were prepared in nuclear extraction buffer as described previously.²⁰ Briefly, protein concentration was measured by Multiskan® Spectrum (Thermo Fisher Scientific, Waltham, MA, USA). Consensus oligonucleotides (nuclear factor-kappa B [NF-κB] and activator protein-1 [AP-1]; Promega Corporation, Fitchburg, WI,

USA) were end-labeled with [γ - 32 P] ATP 3,000 Ci/mmol (Dupont NEN Research Products, Boston, MA, USA). Nuclear proteins (5 μ g) were incubated with a labeled oligonucleotide probe in binding buffer at room temperature for 30 minutes, and then separated in 5% nondenaturing polyacrylamide gel (Acrylamide/Bis-acrylamide at a ratio of 29:1, 50 mM Tris-HCl, 380 mM glycine, 2 mM ethylenediaminetetraacetic acid [EDTA], 2.5% glycerol). The dried gel was exposed to autoradiography film at -80°C with enhancer foils.

ELISA analysis

Supernatants from plaques were analyzed with ELISA kits (Roche and eBioscience, San Diego, CA, USA) as described by the manufacturer's specifications.

Western blot

Carotid plaques were cultured as described above. After indicated periods of culture, plaque tissues were smashed and lysated in lysis buffer containing 1% sodium dodecyl sulfate, 1 mM EDTA/ethylene glycol tetraacetic acid, 10 $\mu\text{L}/\text{mL}$ phosphatase inhibitor cocktail 2/3 (Sigma-Aldrich), and one protease inhibitor cocktail tablet (Roche). The supernatant was recovered by centrifugation at 14,000 rpm at 4°C for 5 minutes. Removal of impurities from the supernatant was implemented with 0.65 μm and 0.1 μm centrifugal filter devices (Millipore Corporation, Billerica, MA, USA). The protein concentration was determined using a Bio-Rad Protein Assay kit (Hercules, CA, USA). A total of 40 μg of each lysate was run on a 4%–12% Bis-Tris gel (Life Technologies), followed by protein transfer to a polyvinylidene fluoride transfer membrane (GE Healthcare Europe GmbH, Freiburg, Germany) using an XCell II™ Blot Module (Life Technologies) according to the manufacturer's instructions. The membrane was incubated with primary antibodies (for phosphorylated extracellular signal-regulated kinase 1/2 [ERK1/2], phosphorylated I kappa B [IkB], glyceraldehyde 3-phosphate dehydrogenase from Cell Signaling Technology [Danvers, MA, USA] and Santa Cruz [Dallas, TX, USA]) followed by secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology and Santa Cruz). Protein bands were detected by ECL Western Blotting Substrate (Pierce, Rockford, IL, USA) followed by exposure to X-ray film.

RNA isolation, complementary DNA synthesis, and qPCR

Total cellular RNA was isolated from carotid plaques and immune cells with RNeasy mini kit (QIAGEN, Venlo,

the Netherlands) as described previously.²⁰ Complementary DNA (cDNA) was generated using the First Strand cDNA Synthesis kit (Thermo Fisher Scientific) for reverse transcription according to the manufacturer's instructions. qPCR was performed using the Light Cycler System (Roche) with SYBR Green (LONZA, Basel, Switzerland), Master Mix (Thermo Fisher Scientific) and primers in a final volume of 20 μL . Primer sequences are given in Table 1. Data were analyzed with the relative expression method (the difference in threshold cycle between the target gene and beta-actin as a control).

Statistical analysis

Data analysis was performed using Prism software (Graphpad, La Jolla, CA, USA). For comparison of two groups, the nonparametric Mann–Whitney *U* test was used; for comparison of three or more groups, ANOVA was done with post hoc Tukey's testing. A level of $P < 0.05$ was considered statistically significant.

Table 1 Primer sequences, sense and antisense, optimal temperatures, and specificity of primers and probes used for qPCR are shown

<i>β-actin</i>		
Sense	5'-AGGATGCAGAAGGAGATCACT-3'	58°C
Antisense	5'-GGGTGTAACGCA ACTAAGTCATAG-3'	
<i>IFNγ</i>		
Sense	5'-TCGGTAACTGACTTAATGTCCA-3'	57°C
Antisense	5'-TCCTTTTTCGCTTCCCTGTTTT-3'	
<i>TF</i>		
Sense	5'-TACTTGGCAGGGTCTTCTC-3'	58°C
Antisense	5'-TCACATTCACTTTTGTCCCAC-3'	
<i>IL6</i>		
Sense	5'-AAATTCGGTACATCCTCGACGG-3'	58°C
Antisense	5'-GGAAGGTTCAAGTTGTTTTCTGC-3'	
<i>TNFα</i>		
Sense	5'-TCTTCTCGAACCCCGAGTGA-3'	58°C
Antisense	5'-CCTCTGATGGCACCACCAG-3'	
<i>CCL2 (MCP-1)</i>		
Sense	5'-ATGAAAGTCTCTGCCGCCCTTCT-3'	58°C
Antisense	5'-TGAGTGTTCAAGTCTTCGGAGTT-3'	
<i>ICAM1</i>		
Sense	5'-GGCCTTATTCTCCCTTCC-3'	58°C
Antisense	5'-GGCATAGCTTGGGCATATTC-3'	
<i>IL10</i>		
Sense	5'-TCAAGGCGCATGTGAACTCC-3'	57°C
Antisense	5'-GATGTCAAACCTCACTCATGGCT-3'	

Abbreviations: *β -actin*, beta-actin; *CCL2*, chemokine (C-C motif) ligand 2; *ICAM1*, intercellular adhesion molecule-1; *IFN γ* , interferon gamma; *IL6*, interleukin-6; *IL10*, interleukin-10; *MCP-1*, monocyte chemoattractant protein-1; qPCR, quantitative polymerase chain reaction; *TF*, tissue factor; *TNF α* , tumor necrosis factor alpha.

Results

Inhibitory role of RhuDex® on LPS-induced activation of the inflammatory milieu in atherosclerotic lesions

Stimulation of plaque tissue with LPS resulted in significantly higher protein levels of TNF- α after 3 hours and 8 hours and IFN- γ after 8 hours in the supernatant (Figure 1A–D). Similarly, protein levels of the chemokine MCP-1, primarily expressed by APCs, in the supernatant of LPS-stimulated plaques were also significantly upregulated at indicated time points (Figure 1E and F). Plaque pieces incubated with LPS in addition to RhuDex® showed a total inhibition of LPS-induced upregulation of TNF- α expression after 3 hours and 8 hours and IFN- γ after 8 hours (Figure 1A–D). Serum protein levels of MCP-1 showed no significant difference between LPS and LPS in addition to RhuDex® after both time points (Figure 1E and F).

To evaluate whether the downregulation of TNF- α and IFN- γ is triggered by an upregulation of anti-inflammatory cytokine IL-10, we measured the levels of the molecule in the supernatant. Interestingly, the study demonstrated that RhuDex® significantly inhibited LPS-induced upregulation of IL-10 in the supernatant of plaques incubated with LPS and RhuDex® after 3 hours and no difference after 8 hours (Figure 1G and H).

qPCR results of cultured plaques underlined ELISA analysis showing an inhibitory effect of RhuDex® on LPS-induced inflammatory response in atherosclerotic lesions. Messenger RNA (mRNA) expression levels of cytokine TNF α were highly upregulated after 3 hours and 8 hours of LPS stimulation (Table 2), whereas IFN γ showed only a significant increase after 8 hours (Table 2). Besides molecules predominantly expressed by T-cells, LPS stimulation of plaque tissues displayed a significant increase of intercellular adhesion molecule-1 (ICAM1), involved in atherogenesis, and prothrombotic molecule tissue factor (TF) after 3 hours and 8 hours (Table 2). LPS in addition to RhuDex® resulted in a significant inhibition of mRNA level upregulation of TNF α , IFN γ , ICAM1, and TF after 3 hours and 8 hours compared to LPS alone (Table 2).

Notably, no difference was found for RhuDex® alone versus unstimulated plaque pieces according to the measured molecules, respectively.

Effects of RhuDex® on LPS-induced cell activation in atherosclerotic lesions are independent of patients' symptoms and plaque morphology

In order to analyze whether plaque pieces from patients with or without ischemic symptoms (stroke or TIA) or the plaque

Figure 1 Protein levels of cytokines and chemokines from the supernatant of cultured carotid artery plaques.

Notes: Plaque tissue samples were randomly planted into a 48-well plate with RPMI medium and then stimulated with LPS (1 μ g/mL), LPS plus RhuDex® (Medigene AG, Planegg/Martinsried, Germany), or RhuDex® (3 μ g/mL) for 3 hours or 8 hours. Unstimulated plaque pieces served as controls. Supernatants were collected for measurement of protein concentrations by ELISA after indicated time. The results shown represent the average of six independent experiments. Results are shown as box plots displaying mean and 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers. #Represents versus unstimulated $P < 0.005$; *represents versus LPS $P < 0.009$.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; h, hours; IFN- γ , interferon gamma; IL-10, interleukin-10; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor alpha; RPMI, Roswell Park Memorial Institute.

Table 2 Quantitative plaque tissue RT-PCR results for different cytokines, adhesion molecules, and prothrombotic molecules

Variable	Unstimulated	LPS	LPS + RhuDex®	RhuDex®
3 hours				
<i>TNFα</i>	4.2±4	21.3±15*	4.0±2†	3.4±2‡
<i>IFNγ</i>	37.3±42	39.7±56	14.4±7	17.1±14
<i>ICAM1</i>	64.9±25	193.7±86*	102.0±92	68.5±33‡
<i>TF</i>	4.2±2	9.2±4*	8.3±4	4.1±2‡
8 hours				
<i>TNFα</i>	6.6±6	14.0±9*	3.9±2†	5.8±5‡
<i>IFNγ</i>	18±9	86.4±41*	34.2±23†	28.3±19‡
<i>ICAM1</i>	147.6±77	478.6±254*	153.5±105†	103.8±50‡
<i>TF</i>	7.2±3	14.4±5*	4.6±1†	5.9±3‡

Notes: Values are normalized to β-actin and expressed as cDNA copies/1,000 β-actin copies. All values are shown as mean ± SD. RhuDex® (Medigene AG, Planegg/Martinsried, Germany). *versus unstimulated $P<0.001$; †versus LPS $P<0.01$; ‡versus LPS $P<0.04$.

Abbreviations: β-actin, beta-actin; cDNA, complementary DNA; *ICAM1*, intercellular adhesion molecule-1; *IFNγ*, interferon gamma; LPS, lipopolysaccharides; ns, not significant; RT-PCR, polymerase chain reaction; SD, standard deviation; *TF*, tissue factor; *TNFα*, tumor necrosis factor alpha.

morphology (complicated lesion versus lipid-rich lesion) influenced the inhibitory functions of RhuDex®, we grouped the plaque pieces according to symptomatic patients and type of plaque. The study demonstrated that neither the symptoms nor plaque morphology had an effect on the inhibitory function of RhuDex® on LPS-induced plaque activation (plaque morphology, Table 3; with or without ischemic symptoms, data not shown).

Effects of RhuDex® on LPS-induced T-cell activation in vitro

To further investigate the specific inhibitory effects of B7-1 by RhuDex®, we stimulated monocytes cocultured with T-cells with LPS. qPCR results of cultured plaques displayed

that RhuDex® had an inhibitory effect on LPS-induced upregulation of proinflammatory cytokines *TNFα* and *IFNγ*, both expressed by T-cells, and chemokine *CCL2* (Figure 2A–E). RhuDex® had no effect on LPS-induced upregulation of *IL6* and *IL10* (Figure 2C and D).

RhuDex® inhibits the LPS-induced expression of cytokines and chemokines in different inflammatory cells in vitro

Since RhuDex® inhibited not only the expression of T-cell activation dependent molecules such as *TNFα* and *IFNγ*, but also of *ICAM1* and *TF*, we further studied specific effects of this drug on monocytes and macrophages in vitro.

Table 3 Quantitative plaque tissue RT-PCR results for different cytokines, adhesion molecules, and prothrombotic molecules according to plaque morphology (complicated lesion versus lipid-rich lesion)

Variable	Unstimulated	LPS	LPS + RhuDex®	RhuDex®
3 hours				
<i>TNFα</i>				
Complicated	4.1±4	20.7±22	3.5±2	2.9±1
Lipid rich	4.3±5	21.8±13	4.3±2	3.5±2
<i>IFNγ</i>				
Complicated	33.5±15	13.5±5	12.7±13	9.6±8
Lipid rich	39.9±56	57.2±70	16.0±3	22.1±19
<i>ICAM1</i>				
Complicated	60.8±23	151.4±59	95.7±25	74.5±30
Lipid rich	67.6±28	221.9±94	109.6±48	62.4±36
<i>TF</i>				
Complicated	5.0±3	6.5±3	8.5±4	5.6±3
Lipid rich	3.5±2	11.0±5	8.2±4	3.1±1

Notes: Values are normalized to β-actin and expressed as cDNA copies/1,000 β-actin copies. All values are shown as mean ± SD. RhuDex® (Medigene AG, Planegg/Martinsried, Germany).

Abbreviations: β-actin, beta actin; cDNA, complementary DNA; *ICAM1*, intercellular adhesion molecule-1; *IFNγ*, interferon gamma; LPS, lipopolysaccharides; ns, not significant; RT-PCR, polymerase chain reaction; SD, standard deviation; *TF*, tissue factor; *TNFα*, tumor necrosis factor alpha.

Figure 2 Effects of RhuDex® LPS-dependent monocyte and T-cell activation in vitro.

Notes: Monocytes and CD4⁺ T-cells from the same donor were isolated from PBMCs and planted in a 6-well plate. Cells were stimulated with LPS (1 µg/mL), LPS plus RhuDex® (Medigene AG, Planegg/Martinsried, Germany) (3 µg/mL), or RhuDex® for 3 hours. Unstimulated monocytes and T-cells served as controls. After an indicated period, cells were collected for RNA extraction. PCR was used to analyze mRNA expression of *TNFα* (A), *IFNγ* (B), *IL-10* (C), *IL-6* (D), and *CCL2* (E). Results were normalized to β-actin. The results shown represent the average of six independent experiments. Results are shown as box plots displaying mean and 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers. #Represents versus unstimulated $P < 0.002$; *represents versus LPS $P < 0.01$; +represents versus LPS $P = 0.01$.

Abbreviations: β-actin, beta-actin; *CCL2*, chemokine (C-C motif) ligand 2; *IFNγ*, interferon gamma; *IL-6*, interleukin-6; *IL-10*, interleukin-10; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein-1; mRNA, messenger; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; *TNFα*, tumor necrosis factor.

Monocytes

To further evaluate the origin of molecular expression, we stimulated human blood-derived monocytes and macrophages with LPS (in addition to RhuDex®) for 3 hours. Incubation of monocytes with LPS and RhuDex® resulted in significant lower mRNA expression levels of cytokines *TNFα*, *IFNγ*, *IL6*, and *IL10* as well as the chemokine *CCL2* (MCP-1) compared to LPS alone (Figure 3A–E).

Macrophages

Monocyte-derived macrophages were also stimulated with LPS (in addition to RhuDex®) for 3 hours. qPCR analysis demonstrated that coinubation of macrophages with LPS and RhuDex® resulted in reduced expression of *TNFα*, *IFNγ*, and *IL10* than LPS alone (data not shown). No inhibitory effect was observed on *CCL2* (MCP-1) and *IL6* expression (data not shown).

RhuDex® modulates expression of cytokines and chemokines by inhibiting NF-κB-, AP-1-, and ERK1/2-related signaling pathways

In order to further analyze how the effect of RhuDex® is mediated, we investigated the signal cascade NF-κB, ERK1/2,

and signal transducer and activator of transcription (STAT)4, known to be involved in the expression of *IFN-γ*, *TNF-α*, *IL-6*, *IL-10*, and MCP-1 ex vivo. Using electrophoretic mobility shift assay of nuclear extracts, the study revealed a significant decrease in the activity of NF-κB and AP-1 in the LPS and RhuDex® group compared to the LPS group after 3 hours of stimulation ex vivo and in vitro (ex vivo Figure 4A and B; in vitro not shown). Furthermore, results of Western blot further demonstrated a significant reduction of phosphorylated IκB, whereas IκB was significantly increased (Figure 4C). In addition, activation of ERK1/2 was also detected and showed that phosphorylated ERK1/2 in relation to total ERK1/2 was markedly reduced by RhuDex® in addition to LPS compared to LPS alone. This resulted in an inactivation of AP-1 (Figure 4D). Notably, no difference was found for STAT4 between the groups (data not shown).

Discussion

It is well known that atherosclerosis is a chronic, (auto) immune disease,^{2,21} and that T-cells and APCs are mainly involved in atherogenesis.²¹

RhuDex® as a specific B7-1 inhibitor is able to inhibit LPS-mediated plaque tissue inflammation and cytokine

Figure 3 In vitro effects of RhuDex® on monocyte activation.

Notes: Monocytes were isolated from PBMCs and planted in a 6-well plate. Cells were stimulated with LPS (1 µg/mL), LPS plus RhuDex® (Medigene AG, Planegg/Martinsried, Germany), or RhuDex® (3 µg/mL) for 3 hours. Unstimulated monocytes served as controls. After an indicated period, cells were collected for RNA extraction. PCR was used to analyze mRNA expression of *TNFα* (A), *IFNγ* (B), *IL10* (C), *IL6* (D), and *CCL2* (E). Results were normalized to β-actin; The results shown represent the average of six independent experiments. All values are shown as column bar graphs and plots represent mean ± SD. [#]Represents versus unstimulated $P < 0.01$; ^{*}represents versus LPS $P < 0.05$.

Abbreviations: β-actin, beta-actin; *CCL2*, chemokine (C-C motif) ligand 2; *IFNγ*, interferon gamma; *IL6*, interleukin-6; *IL10*, interleukin-10; LPS, lipopolysaccharides; mRNA, messenger RNA; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; SD, standard deviation; *TNFα*, tumor necrosis factor.

(TNF-α, IFN-γ, IL-6), chemokine (MCP-1), adhesion molecule (ICAM1), and prothrombotic molecule (TF) expression in atherosclerotic lesions. These results are found to be independent of the lesion type (stable and vulnerable) and symptoms of patients (stroke and TIA versus no symptoms). RhuDex® not only inhibited T-cell activation but also negatively influenced APC activation. The effects of RhuDex® are due to a reduced expression of atherogenic promoters by downregulating phosphorylation of ERK1/2 and transcription factors NF-κB/AP-1.

In order to evaluate the impact of the specific B7-1 inhibitor RhuDex® on the lesional cellular compound, we evaluated LPS as an adequate stimulator. LPS is a potent toll-like receptor (TLR)4 signaling activator and already described to activate the inflammatory compound in human atherosclerotic lesions *ex vivo*.²² The pathophysiologic aspect of TLR4 in atherogenesis relates to its additional ligands, known autoantigens in atherogenesis such as heat shock protein 60 from *Chlamydia pneumoniae* or oxidized low density lipoprotein. In addition, TLR expression is upregulated in macrophages and endothelial cells in human

atherosclerotic plaques, and their signaling has implications for lesion development, foam cell formation, inflammation, matrix degradation, and ischemia-reperfusion.^{23–25}

Moreover, B7-1 is constantly expressed on different atherogenic cell types such as monocytes and macrophages, and LPS is known to upregulate this costimulatory molecule.^{26–28} By inducing APCs due to LPS, these cells are capable of activating T-cells. This step is major histocompatibility complex-unrestricted but is strongly dependent on interactions of CD28 and/or CTLA-4 on T-cells and their ligands B7-1/B7-2 on APCs.²⁹ The current study is in line with previous results showing that LPS activation induced an upregulation of the expression of various inflammatory molecules in cultured plaques as well as in monocytes and macrophages, which are known to be primarily involved in atherogenesis.^{30–34} Thus, it can be concluded that LPS represents a good candidate to investigate the role of B7-1 on lesional inflammation.

Various cytokines and chemokines are involved in atherogenesis. TNF-α, IFN-γ, and IL-6 are major proinflammatory cytokines, known to promote atherosclerosis

Figure 4 Effects of RhuDex® on LPS-induced lesion inflammation is mediated by several signaling pathways.

Notes: Cultured carotid artery plaque pieces were incubated with LPS (1 µg/mL) or LPS plus RhuDex® (Medigene AG, Planegg/Martinsried, Germany) (3 µg/mL) for 3 hours, and then prepared for the extraction of nuclear proteins. Specimen were smashed and lysed for protein isolation. Unstimulated plaque pieces served as controls. Proteins were labeled with radioactive reagents for an indicated time and exposed to detect NF-κB and AP-1 levels by EMSA or analyzed by western blotting (phosphorylated ERK 1/2, phosphorylated IκB and IκB). The results shown represent the average of six independent experiments, representative EMSA (A and B) and western blot (C and D) together with its quantitative analysis are shown. All values are shown as column bar graphs and plots represent mean ± SD. [#]versus unstimulated, $P < 0.03$; ^{*}versus LPS, $P < 0.05$.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharides; NF-κB, nuclear factor-kappa B; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; ERK 1/2, extracellular signal-regulated kinase; IκB, I kappa B; P-ERK 1/2, phosphorylated; P-IκB, phosphorylated I kappa B; SD, standard deviation.

and plaque instability, whereas anti-inflammatory cytokine IL-10 is known to inhibit plaque development and progression.^{30–33,35–37} ICAM1, as a major adhesion molecule, and MCP-1, as a potent chemoattractant, also display

proatherogenic functions.^{38,39} TF, one of the prothrombotic molecules, is known to be primarily involved in the initiation of a thrombus formation.³⁴ Our study demonstrates that RhuDex® has potent inhibitory effects on the activation of the

inflammatory milieu in atherosclerotic lesions by inhibiting the secretion of proinflammatory mediators induced by LPS stimulation both *ex vivo* and *in vitro*. Interestingly, the effects of RhuDex® were not restricted to T-cell-derived molecular expression but also other cell types such as APCs. Thus, it can be suggested that B7-1 is involved in the inflammatory milieu of an atherosclerotic lesion.

The mechanism by which RhuDex® acts as a specific B7-1 inhibitor is capable of inhibiting APC activation induced by LPS. It is known that *in vitro* regulatory T-cells specifically downregulate the expression of B7-1/B7-2 on DCs in both a CTLA-4- and lymphocyte function-associated antigen-1-dependent manner.⁴⁰ This B7-1/B7-2 downmodulating effect was still present, even in the presence of the strong DC-dependent stimuli of LPS.⁴⁰ This indicates that blockade of B7-1/B7-2 resulted in a negative feedback in APCs. However, it remains unknown whether the grade of APC activation can be also inhibited by the negative feedback. Our study clearly demonstrated that blockade of B7-1 by RhuDex® inhibited the upregulation of various molecules expressed by APCs, independently of interaction with T-cells. Thus, blockade of the B7-1 receptor by RhuDex® inhibited LPS-induced activation of APCs, most likely by a B7-1 receptor-dependent negative feedback in APCs. Additional studies are needed to further investigate the possible negative feedback.

To further evaluate the finding of a general inhibition of the LPS-induced cellular activation by RhuDex®, we focused on IL-10. IL-10 is an anti-inflammatory cytokine produced by T-cells, macrophages, monocytes, and DCs.^{36,37} A potent effect of IL-10 is to inhibit the production of mediators, including TNF- α , IFN- γ , IL-6, and MCP-1.⁴¹ It is further known that IL-10 gene expression is regulated by many transcription factors involving NF- κ B, STAT1/3, AP-1, CCAAT/enhancer binding protein (C/EBP) β and C/EBP δ .^{41–44} Interestingly, the study revealed that upregulation of IL-10 by LPS was also inhibited by RhuDex®. Thus, IL-10 is not responsible for the effects of RhuDex® on cytokine expression. The underlying signaling pathway is at least in part mediated by NF- κ B/AP-1. In conclusion, it can be suggested that the RhuDex®-dependent negatively-influenced cytokine expression seems to be based on a general inhibition of the induction of cellular activation induced by LPS rather than mediated by anti-inflammatory cytokine IL-10 in atherosclerotic lesions.

In evaluating the inhibitory effect on cellular activation and molecular expression by RhuDex®, we used electrophoretic mobility shift assay and western blot to investigate possible signaling cascades. It can be speculated that activation of the lesional cellular compound via LPS further degrades I κ B and

mitogen-activated protein kinases, thereby inducing NF- κ B and mitogen-activated protein kinase-dependent gene transcription as shown in recent publications.^{23,45,46} LPS-induced TNF- α gene expression is dependent on the activation of ERK1/2, whereas MCP-1 is regulated by NF- κ B and ERK1/2-dependent pathways.⁴⁷ An early activation of AP-1 and NF- κ B further leads to a significant increase in IL-6 gene expression, and IFN- γ production is regulated by a variety of transcription factors.^{48,49} Subsequently, this may lead to enhanced inflammation by triggering the induction of additional adhesion molecules, cytokines, and growth factors. The current findings demonstrate that the inhibitory effect of RhuDex® on cellular activation and expression of various molecules are likely due to the inhibition of NF- κ B and ERK1/2 pathways.

The contribution of B7-1 and B7-2 costimulation to immune responses in atherosclerotic lesions remains controversial and illustrates the complexity of these pathways. Buono et al showed that cholesterol diet-fed B7-1^{-/-}/B7-2^{-/-} Ldlr^{-/-} mice had significantly reduced atherosclerotic lesion development.¹⁴ The B7-1^{-/-}/B7-2^{-/-} Ldlr^{-/-} knockout resulted in less IFN- γ production of CD4⁺ T-cells in response to the TLR4 ligand heat shock protein 60 *in vitro*.¹⁴ In contrast, Ait-Oufella et al showed that irradiated bone marrow chimeric Ldlr^{-/-} mice reconstituted with B7-1^{-/-}/B7-2^{-/-}, CD28^{-/-} resulted in increased atherosclerotic lesion development. These changes were based on an impaired regulatory T-cell development and an enhanced proatherogenic effector T-cell response.¹⁵ However, these findings are derived from mouse studies while only few studies examined the presence of B7-1 and B7-2 in human atherogenesis. de Boer et al demonstrated that the expression of B7-1 and B7-2 was highest on macrophages in the superficial layers of the intima from human arterial segments.¹⁶ We previously found that B7-2 was expressed markedly higher in carotid artery plaques of patients with ischemic symptoms such as stroke or TIA.⁴ The current study clearly demonstrates that B7-1 seems to be a major proinflammatory component in the cascade initiating an innate and adaptive immune response inside atherosclerotic lesions. Thus, B7-1 seems to be primarily a proatherogenic mediator in the inflammatory process in human atherosclerotic lesions and may be an interesting target of future therapeutic agents *in vivo*.

It was not the intention of the study to compare the effects of RhuDex® in atherosclerotic lesions of symptomatic and asymptomatic patients or on plaque morphology. However, the results of the study show that the stimulatory capacity of LPS is comparable between both groups (data not shown). In addition, the inhibitory functions of RhuDex® showed no

difference according to patients with or without ischemic symptoms or plaque morphology. Thus, the role of RhuDex® on the inflammatory response to TLR4 ligand LPS is comparable between atherosclerotic lesions of patients with ischemic symptoms and plaque morphology.

In conclusion, RhuDex® as a specific B7-1 inhibitor appears to show a universal inhibitory effect on LPS-induced inflammation in atherosclerotic lesions. The finding of a possible B7-1-dependent negative feedback on APCs reflects a promising new field of investigation. Therefore, due to the strong anti-inflammatory effects against TLR4-induced lesional cell activation by LPS, RhuDex® seems to have the potential capability to be a promising anti-inflammatory agent, which provides a novel potential therapeutic prospect of atherosclerosis.

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Disclosure

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