Inhibition of Arthritis in the Lewis Rat by Apolipoprotein A-I and Reconstituted High-Density Lipoproteins

Ben J. Wu, Kwok L. Ong, Sudichhya Shrestha, Kang Chen, Fatiha Tabet, Philip J. Barter, Kerry-Anne Rye

Objective—This study shows whether high-density lipoproteins (HDLs) and apolipoprotein A-I inhibit joint inflammation in streptococcal cell wall peptidoglycan-polysaccharide (PG-PS)–induced arthritis in female Lewis rats.

Approach and Results—Administration of PG-PS to female Lewis rats caused acute joint inflammation after 4 days, followed by remission by day 8. The animals subsequently developed chronic joint inflammation that persisted until euthanized at day 21. Treatment with apolipoprotein A-I 24 hours before and after PG-PS administration reduced the acute and chronic joint inflammation. Treatment with apolipoprotein A-I at days 7, 9, and 11 after PG-PS administration reduced the chronic joint inflammation. Treatment with apolipoprotein A-I for 24 hours before and after PG-PS administration reduced acute and chronic joint inflammation. Treatment with apolipoprotein A-I also reduced the inflammatory white blood cell count, synovial fluid proinflammatory cytokine levels, synovial tissue macrophage accumulation, as well as Toll-like receptor 2, and inflammatory cytokine expression. At the molecular level, preincubation of human monocyte–derived macrophages with apolipoprotein A-I or reconstituted HDLs before PG-PS stimulation inhibited the PG-PS–induced increase in Toll-like receptor 2 and myeloid differentiation primary response gene (88) mRNA levels, nuclear factor-kB activation, and proinflammatory cytokine production. The effects of apolipoprotein A-I and reconstituted HDLs were abolished by transfecting the human monocyte–derived macrophages with ATP-binding cassette transporter A1 or G1 siRNA.

Conclusions—Apolipoprotein A-I and reconstituted HDLs attenuate PG-PS–induced arthritis in the rat. Studies in human monocyte–derived macrophages indicate that this benefit may be because of the inhibition of Toll-like receptor 2 expression and decreased nuclear factor-kB activation in macrophages. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: apolipoprotein A-I ■ arthritis, rheumatoid ■ cholesterol, HDL ■ inflammation

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes joint destruction and is associated with the migration of inflammatory cells into synovial tissues. Although the causes of RA are unknown, activation of synovial cell toll-like receptors (TLRs) plays a pivotal role in its progression. To date, 10 TLRs have been identified in humans and 12 in mice, including TLR2 that recognizes bacterial components, such as peptidoglycans (PGs). The cytosolic domains of TLRs interact with adaptor proteins, triggering the activation of downstream inflammatory pathways. Myeloid differentiation primary response gene (88) (MyD88), the best characterized TLR adaptor protein, is essential for initiating TLR2 signaling pathways, downstream activation of nuclear factor-kB (NF-kB), and transcription of genes that produce the proinflammatory cytokines, chemokines, and proteases that cause joint inflammation and destruction in arthritis. The high-density lipoproteins (HDLs) in human plasma consist of multiple subpopulations of particles, high levels of which are associated with reduced cardiovascular risk. The most extensively studied function of HDLs and the main HDL apolipoprotein (apolipoprotein A-I) involves their participation in the reverse cholesterol transport pathway, whereby excess cholesterol is exported from peripheral cells and transported to the liver for excretion. These processes are dependent on scavenger receptor scavenger receptor B1 (SR-B1) and the ATP-binding cassette transporters (ABCA1) and ABCG1. HDLs and apolipoprotein A-I are also profoundly anti-inflammatory and inhibit acute and chronic vascular inflammation in vivo. HDL cholesterol and apolipoprotein A-I levels are reduced in people with RA relative to healthy controls. HDL cholesterol levels are also significantly decreased in a retrospective study of blood donors who later developed...
Apolipoprotein A-I protects against peptidoglycan-polysaccharide (PG-PS)–induced arthritis in rats. Female Lewis rats received a single intraperitoneal (ip) injection of saline or PG-PS (15 mg/kg). The control animals received intravenous (iv) injections of saline 24 hours before and on days 1, 7, 9, and 11 after PG-PS injection. The remaining animals received apoA-I (8 mg/kg, iv) 24 hours before and 24 hours after the PG-PS injection, and then apoA-I (8 mg/kg, iv) on days 7, 9, and 11 after PG-PS injection. Arthritis scores were used to assess joint inflammation as described in the Materials and Methods in the online-only Data Supplement. The pictures in A were taken on day 21 immediately before euthanasia. Data are expressed as means±SEM. *P<0.05 vs PG-PS treated, saline (S/S) infused rats.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
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<td>ABCG1</td>
<td>ATP-binding cassette transporter G1</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<td>HMDMs</td>
<td>human monocyte–derived macrophages</td>
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<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response gene (88)</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<tr>
<td>PG-PS</td>
<td>peptidoglycan-polysaccharide</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>rHDL</td>
<td>reconstituted HDL</td>
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<tr>
<td>SR-B1</td>
<td>scavenger receptor B1</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>WBC</td>
<td>white blood cell</td>
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Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Apolipoprotein A-I Prevents PG-PS–Induced Arthritis in Female Lewis Rats

Arthritis was induced in female Lewis rats as described in Materials and Methods in the online-only Data Supplement and Figure I in the online-only Data Supplement. Joint inflammation in the animals that received intravenous saline infusions 24 hours before and at days 1, 7, 9, and 11 after PG-PS administration progressed from an initial, acute inflammatory phase during the first 4 days after PG-PS administration to remission by day 8. These animals subsequently developed chronic joint inflammation that persisted until euthanized at day 21 (Figure 1A–1C, closed circles, S/S). Joint inflammation was not evident in control animals that received intravenous...
saline infusions 24 hours before and at days 1, 7, 9, and 11 after a single intraperitoneal saline injection (Figure 1A, closed triangles, S/S).

Administration of apolipoprotein A-I 24 hours before and 24 hours after the intraperitoneal PG-PS injection, followed by 3 intravenous saline injections on days 7, 9, and 11 after PG-PS injection, decreased the acute and chronic phases of arthritis by 63±9% at day 3 and by 61±8% at day 21 (Figure 1A, open circles, A/I; P<0.05 for both). Infusion of saline 24 hours before and 24 hours after the PG-PS injection, followed by infusion of apolipoprotein A-I on days 7, 9, and 11 after PG-PS injection, had no effect on acute joint inflammation but reduced chronic joint inflammation by 43±11% at day 21 (Figure 1B, open circles, S/AI; P<0.05). Apolipoprotein A-I administration 24 hours before the PG-PS injection, and on days 1, 7, 9, and 11 after the PG-PS injection, reduced joint inflammation by 61±5% at day 3 and by 90±5% at day 21 (Figure 1C, open circles, AI/AI; P<0.05 for both).

**Apolipoprotein A-I Reduces PG-PS–Induced Inflammatory Cell Infiltration and TLR2 Expression in Synovial Tissues**

Joint inflammation in arthritis is mediated by the migration of circulating inflammatory cells into synovial tissues, where they subsequently release proinflammatory cytokines. The present results are consistent with these events. When the animals were treated with intravenous saline on day 1 before and days 1, 7, 9, and 11 after inducing arthritis with an intraperitoneal PG-PS infusion, the total white blood cell (WBC), neutrophil, and monocyte counts increased by 6.1±0.5-, 17±1.4-, and 37±4.9-fold, respectively (Figure 2A–2C, S/S; P<0.05 for all versus saline-treated animals with arthritis). When the animals with PG-PS–induced arthritis were treated with apolipoprotein A-I, the WBC, neutrophil, and monocyte counts decreased by 49% to 64% (Figure 2A–2C, S/AI; P<0.05 vs PG-PS treated, saline (S/S)) in the control animals that received a single intraperitoneal injection of saline and were treated with intravenous saline (Figure 3A–3D, S/S). A single intraperitoneal PG-PS infusion, by contrast, induced massive WBC filtration into the synovial fluid (Figure 3A and 3B, closed bars). The major synovial fluid WBCs in the PG-PS–treated animals were neutrophils, and combined CD11b/c and CD44 expression on circulating WBCs in the saline-treated animals with PG-PS–induced arthritis also increased by 65±6.0-, 55±1.9-, and 128±7.1-fold, respectively (Figure 2D–2F, S/S; P<0.05 for all).

When the animals with PG-PS–induced arthritis were treated with apolipoprotein A-I, the WBC, neutrophil, and monocyte counts decreased by 49% to 64% (Figure 2A–2C, S/AI; P<0.05 vs PG-PS treated, saline (S/S)). Infusion of saline 24 hours before and 24 hours after the PG-PS injection, followed by 3 intravenous saline injections on days 7, 9, and 11 after PG-PS injection, had no effect on acute joint inflammation but reduced chronic joint inflammation by 75% to 92%, 59% to 90%, and 84% to 97%, respectively (P<0.05 for all versus saline-treated animals with arthritis).

The plasma TNF-α concentration that was below the level of detection in the control animals increased to 3.7±0.8 pg/mL in the saline-treated animals with arthritis (Figure 2G). Plasma IL-6 (Figure 2H) and IL-1β (Figure 2I) levels in the saline-treated animals with PG-PS–induced arthritis increased by 2.7±0.3- and 6.5±0.5-fold, respectively (P<0.05 for all compared with animals without arthritis). When the animals with PG-PS–induced arthritis were treated with apolipoprotein A-I, plasma TNF-α levels decreased by 57% to 64% (Figure 2G), IL-6 levels decreased by 42% to 68% (Figure 2H), and IL-1β levels decreased by 40% to 55% (Figure 2I; P<0.05 for all). WBCs were not detected in synovial fluid samples from control animals that received a single intraperitoneal injection of saline and were treated with intravenous saline (Figure 3A–3D, S/S). A single intraperitoneal PG-PS infusion, by contrast, induced massive WBC filtration into the synovial fluid (Figure 3A and 3B, closed bars). The major synovial fluid WBCs in the PG-PS–treated animals were neutrophils. CD11b/c, CD44, and combined CD11b/c and CD44 expression on circulating WBCs in the saline-treated animals with PG-PS–induced arthritis also increased by 65±6.0-, 55±1.9-, and 128±7.1-fold, respectively (Figure 2D–2F, S/S; P<0.05 for all).
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Figure 3. Apolipoprotein A-I (ApoA-I) inhibits the peptidoglycan-polysaccharide (PG-PS)–mediated infiltration of inflammatory white blood cells (WBCs) and proinflammatory cytokines into the synovium. Female Lewis rats received a single intraperitoneal (ip) injection of saline (open bars) or PG-PS (closed bars) and intravenous (iv) injections of saline or apoA-I as described in the legend to Figure 1. Synovial fluid was collected during euthanasia on day 21 after the ip PG-PS or saline injection. A, Synovial fluid WBCs. Total WBCs (B), neutrophils (C), and monocytes (D) in the synovial fluid were determined by manual counting. Synovial fluid tumor necrosis factor (TNF)-α (E), IL-6 (F), and IL-1β (G) levels were determined by ELISA. Data represent means±SEM. *P<0.05 versus PG-PS-treated, saline (S/S) infused rats.

Figure 4. Apolipoprotein A-I (ApoA-I) reduces the peptidoglycan-polysaccharide (PG-PS)–mediated increase in joint macrophage content and synovial tissue (Syn) tumor necrosis factor (TNF)-α and toll-like receptor (TLR) 2 mRNA levels. Female Lewis rats received a single intraperitoneal (ip) injection of saline (open bars) or PG-PS (closed bars) and intravenous (iv) injections of saline or lipid-free apoA-I as described in the legend to Figure 1. Tibiotarsal joint sections and Syn were obtained during euthanasia on day 21 after PG-PS or saline injection. A, Tibiotarsal joint sections immunostained for CD68 (macrophages), and quantified as described in Materials and Methods in the online-only Data Supplement. Synovial tissue TNF-α (B) and TLR2 (C) mRNA levels were quantified by quantitative polymerase chain reaction. Data represent means±SEM. *P<0.05 versus PG-PS-treated, saline (S/S) infused rats.
migration into synovial tissues and the subsequent release of proinflammatory cytokines. These findings also suggest that the ability of apolipoprotein A-I to reduce joint inflammation in rats with PG-PS–induced arthritis may be related to reduced TLR2 expression in macrophages.

Apolipoprotein A-I Inhibits PG-PS–Induced Inflammation in Macrophages by Attenuating TLR2 Expression and Activation

The inhibition of PG-PS–induced proinflammatory cytokine production by apolipoprotein A-I and the involvement of TLR2 in this process was investigated in HMDMs. Stimulation of HMDMs with PG-PS increased TNF-α (Figure 5A, closed bar), IL-6 (Figure 5B, closed bar), and IL-1β (Figure 5C, closed bar) secretion into the culture medium by 8.5±0.2-, 12±0.9-, and 2.8±0.4-fold, respectively (P<0.05 for all versus control). Preincubation of the HMDMs with apolipoprotein A-I at a final concentration of 0.25, 0.5, and 1.0 mg/mL inhibited the PG-PS–mediated increase in TNF-α levels by 38±5.6%, 53±6.8%, and 64±8.1%, respectively (Figure 5A), reduced IL-6 levels by 36±10%, 48±12%, and 55±6.9% (Figure 5B), and decreased IL-1β levels by 34±9.4%, 53±9.1%, and 60±1.0% (Figure 5C; P<0.05 for all).

The ability of apolipoprotein A-I to prevent the PG-PS–mediated increase in TLR2 and MyD88 expression and downstream activation of NF-κB in HMDMs was also investigated. Relative to what was observed for control HMDMs (open bars), incubation with PG-PS increased TLR2 (Figure 5D) and MyD88 (Figure 5E) mRNA levels 3.0±0.3- and 2.9±0.2-fold, respectively (closed bars), whereas nuclear levels of the p65 subunit of NF-κB (Figure 5E, closed bars) increased by 8.2±1.0-fold (P<0.05 for all). Preincubation of HMDMs with apolipoprotein A-I (final concentration 0.25, 0.5, and 1.0 mg/mL) inhibited the PG-PS–mediated increase in TLR2 mRNA levels by 33±3.4%, 48±1.9%, and 59±6.6% (Figure 5D), reduced MyD88 mRNA levels by 32±7.3%, 50±12%, and 62±13% (Figure 5E), and decreased nuclear p65 protein levels by 38±2.3%, 50±8.7%, and 69±8.1% (Figure 5F; P<0.05 for all). When taken together, these results indicate that apolipoprotein A-I inhibits PG-PS–induced inflammation in macrophages by inhibiting TLR2 and MyD88 gene expression and downstream activation of NF-κB.

To confirm that apolipoprotein A-I inhibited the PG-PS–mediated activation of HMDMs in a TLR2-dependent manner, HMDMs were transfected with TLR2 siRNA. TLR2 protein (Figure IIA in the online-only Data Supplement) and mRNA (Figure IIB in the online-only Data Supplement) levels in the transfected cells were decreased by 81±10% and by 71±11%, respectively (P<0.05 for both), relative to HMDMs transfected with scrambled siRNA.

When the scrambled siRNA-transfected cells were incubated with PG-PS, HMDM TLR2 mRNA levels increased by 2.9±0.5-fold (Figure IIC in the online-only Data Supplement, open bars; P<0.05). Preincubation of the scrambled siRNA-transfected cells with apolipoprotein A-I before stimulation with PG-PS decreased HMDM TLR2 mRNA levels by 58±3.8% (Figure IIC in the online-only Data Supplement, open bars; P<0.05 relative to PG-PS–treated cells preincubated with PBS). Incubation with PG-PS increased TLR2 mRNA levels in the TLR2 siRNA-transfected cells by 2.6±0.4-fold (P<0.05; Figure IID in the online-only Data Supplement, closed bars). TLR2 mRNA levels were similarly increased when the cells were preincubated with apolipoprotein A-I before stimulation with PG-PS.

Incubation of scrambled siRNA-transfected HMDMs with PG-PS increased the concentration of TNF-α in the culture medium from 0.09±0.02 to 0.88±0.07 ng/mL (Figure IID in the online-only Data Supplement, open bars). Preincubation of the scrambled siRNA-transfected HMDMs with apolipoprotein A-I before stimulation with PG-PS decreased the TNF-α concentration in the medium by 61±11% (Figure IID in the online-only Data Supplement; P<0.05 versus PG-PS–treated cells preincubated with PBS).

TNF-α was not detected in the medium when HMDMs were transfected with TLR2 siRNA (Figure IID in the online-only Data Supplement, closed bars). Preincubation of the TLR2 siRNA-transfected HMDMs with PBS or apolipoprotein A-I...
before stimulation with PG-PS increased the concentration of TNF-α in the medium to 0.16±0.02 and 0.11±0.01 ng/mL, respectively (Figure IID in the online-only Data Supplement).

**Apolipoprotein A-I Decreases PG-PS–Induced TLR2 and NF-kB Activation in Macrophages in an ABCA1-Dependent Manner**

To investigate the mechanism by which apolipoprotein A-I prevents the PG-PS–induced increase in TLR2 and MyD88 mRNA levels and nuclear translocation of NF-kB, HMDMs were transfected with ABCA1 siRNA, ABCG1 siRNA, SR-B1 siRNA, and scrambled siRNA (siControl) for 24, 48, and 72 hours. ABCA1 protein levels were reduced by 29±18%, 76±9.9% (P<0.05), and 75±13%, respectively, after 24, 48, and 72 hours of transfection with ABCA1 siRNA (Figure IIIA in the online-only Data Supplement). Transfection of HMDMs for 24, 48, and 72 hours with ABCG1 siRNA decreased ABCG1 protein levels by 48±9.0%, 69±9.3%, and 77±7.3%, respectively, (Figure IIIB in the online-only Data Supplement; P<0.05 for all). SR-B1 protein levels were decreased by 70±11% after 48 hours and by 89±5.0% after 72 hours of transfection with SR-B1 siRNA (Figure IIC in the online-only Data Supplement; P<0.05 for both). ABCA1, ABCG1, and SR-B1 mRNA levels were decreased by 71±8.4%, 62±9.1%, and 64±7.8% after 48 hours of transfection with their respective siRNAs (Figure IID–IIIIF in the online-only Data Supplement; P<0.05 for all).

Incubation of the scrambled siRNA-transfected HMDMs with PG-PS increased TLR2 mRNA levels 3±0.4-fold (Figure 6A, open bars; P<0.05 relative to control). A similar increase in TLR2 mRNA levels was also observed in HMDMs that were transfected with ABCA1 siRNA, ABCG1 siRNA, and SR-B1 siRNA and incubated with PBS before stimulation with PG-PS (Figure 6A, closed bars). This indicates that the PG-PS–induced expression of TLR2 in HMDMs is independent of ABCA1, ABCG1, and SR-B1. Preincubation of the scrambled siRNA-transfected cells with apolipoprotein A-I inhibited the PG-PS–mediated increase in TLR2 mRNA levels by 53±6.9% (Figure 6A, open bars; P<0.05). TLR2 mRNA levels were similarly inhibited in HMDMs transfected with ABCG1 siRNA and SR-B1 siRNA then preincubated with apolipoprotein A-I before stimulation with PG-PS. Preincubation with apolipoprotein A-I, by contrast, did not inhibit the PG-PS–mediated increase in TLR2 mRNA levels in HMDMs transfected with ABCA1 siRNA (Figure 6A, closed bars). This indicates that the ability of apolipoprotein A-I to inhibit the PG-PS–mediated increase in TLR2 mRNA levels in HMDMs is dependent on ABCA1 but not on ABCG1 or SR-B1.

To determine whether apolipoprotein A-I also inhibits the PG-PS–mediated increase in HMDM MyD88 mRNA levels, nuclear p65 levels, and TNF-α secretion in an ABCA1-dependent manner, HMDMs were transfected with scrambled siRNA and ABCA1 siRNA. Incubation with PG-PS increased MyD88 levels (Figure 6B), nuclear p65 levels (Figure 6C), and TNF-α secretion (Figure 6D) in HMDMs transfected with scrambled siRNA (open bars) and ABCA1 siRNA (closed bars). Preincubation of the scrambled siRNA-transfected HMDMs with apolipoprotein A-I inhibited the PG-PS–mediated increase in MyD88 mRNA levels (Figure 6B, open bars), nuclear p65 expression (Figure 6C, open bars), and TNF-α secretion (Figure 6D, open bars) by 57±9.4%, 74±14%, and 62±12%, respectively (P<0.05 for all). When the PG-PS–treated HMDMs were transfected with ABCA1 siRNA, apolipoprotein A-I was no longer able to inhibit the PG-PS–induced increase in TLR2 mRNA levels (Figure 6B, closed bars), nuclear p65 levels (Figure 6C, closed bars), or TNF-α secretion (Figure 6D, closed bars).

**rHDLs Inhibit PG-PS–Induced TLR2 Expression and NF-kB Activation in Macrophages and Protect Against PG-PS–Induced Arthritis in Lewis Rats**

The ability of CSL-111, an rHDL preparation from CSL Ltd, Australia, to protect against PG-PS–induced arthritis in female Lewis rats was also investigated. The rationale for this experiment was that the longer plasma residence time of CSL-111 relative to that of apolipoprotein A-I may lead to more effective inhibition of joint inflammation, TLR2 expression, and NF-kB activation in HMDMs.20

Incubation of HMDMs with PG-PS increased TLR2 mRNA (Figure IVA in the online-only Data Supplement), MyD88 mRNA (Figure IVB in the online-only Data Supplement), and nuclear NF-kB p65 subunit protein (Figure IVC in the
This report demonstrates for the first time that apolipoprotein inflammation is well established in vitro and in vivo. The ability of apolipoprotein A-I and HDL to inhibit vascular inflammation is well established in vitro and in vivo. Comparison of the results in Figure 1 and Figure VI in the online-only Data Supplement indicate that this is not the case. This unexpected result may be a consequence of association with lipid markedly altering the secondary structure of the C-terminal domain of apolipoprotein A-I in CSL-111 and decreasing surface exposure of specific apolipoprotein A-I residues or peptide sequences with anti-inflammatory or chemotactic properties.

Given that recruitment of circulating leukocytes is a key event in the pathogenesis of joint inflammation in RA, it is possible that the apolipoprotein A-I-mediated reduction in circulating inflammatory cell levels may have been directly responsible for the decreased number of inflammatory cells and the reduction in proinflammatory cytokine levels in the joints of the PG-PS-treated animals. As HDLs from mice transgenic for human apolipoprotein A-I have recently been shown to suppress myeloid cell proliferation, it is also possible that the reduced number of circulating inflammatory cells in the PG-PS-treated animals may have been caused by an apolipoprotein A-I-mediated decrease in leukocyte production. It is also conceivable that apolipoprotein A-I may have crossed the synovial membrane and inhibited macrophage proinflammatory cytokine production in the joints of the PG-PS-treated animals directly.

The finding that apolipoprotein A-I inhibits PG-PS-induced inflammation in HMDMs by decreasing TLR2 and MyD88 mRNA levels and inhibiting NF-κB activation in an ABCA1-dependent manner convincingly demonstrates that the previously reported anti-inflammatory properties of apolipoprotein A-I and HDLs extend beyond their potential MyD88 mRNA levels and inhibiting NF-κB activation in a more effective inhibitor of joint inflammation when compared with apolipoprotein A-I. Comparison of this approach would have the added benefit of reducing cardiovascular risk that is known to be elevated in people with RA.

The observations that apolipoprotein A-I inhibits PG-PS-induced inflammatory responses by reducing TLR2 and MyD88 mRNA levels and inhibiting NF-κB activation in macrophages are consistent with earlier reports showing that the anti-inflammatory effects of apolipoprotein A-I and HDLs are related, at least in part, to decreased TLR signaling in a cardiovascular setting. In those studies, incubation of apolipoprotein A-I or HDLs with macrophages reduced TLR4 expression and prevented the TLR ligand, LPS, from inducing TLR signaling. The results of the present study confirm that these beneficial effects of apolipoprotein A-I and to a lesser extent the rHDL preparation, CSL-111, also inhibit joint inflammation in female Lewis rats with PG-PS-induced arthritis.

As CSL-111 is likely to be cleared from the circulation less rapidly than lipid-free apolipoprotein A-I, we anticipated that it would be a more effective inhibitor of joint inflammation in vivo was also determined. Arthritis was induced in female Lewis rats with a single intraperitoneal infusion of PG-PS. The animals also received intravenous injections of saline or CSL-111 (Figure VI in the online-only Data Supplement, closed circles) or CSL-111 (Figure VI in the online-only Data Supplement, open circles) 24 hours before and at days 1, 7, 9, and 11 after the PG-PS injection. Treatment with CSL-111 reduced the acute and chronic phases of joint inflammation by 44±4% at day 3 and by 43±9% at day 21 (Figure VI in the online-only Data Supplement, open circles; P<0.05 for both).

Discussion

The ability of apolipoprotein A-I and HDL to inhibit vascular inflammation is well established in vitro and in vivo. This report demonstrates for the first time that apolipoprotein A-I and to a lesser extent the rHDL preparation, CSL-111, also inhibit joint inflammation in female Lewis rats with PG-PS-induced arthritis.
apolipoprotein A-I are also apparent in an entirely different pathology. Apolipoprotein A-I and the apolipoprotein A-I mimetic peptide, 4F, have also been reported to decrease TLR4 expression and protect against LPS-induced inflammation in HMDMs. Similary, the increased HDL levels in mice transgenic for human apolipoprotein A-I are associated with decreased TLR4 expression in the lung and protect against LPS-induced lung inflammation. 20

The current results showing that CSL-111 and apolipoprotein A-I inhibit macrophage and joint inflammation by decreasing TLR expression and signaling, as well as the expression of inflammatory genes in an ABCA1- and ABCG1-dependent manner, are consistent with what was reported for the first time in 2008 by Yvan-Charvet et al, 32 who found that ABCA1-ABCG1-deficient macrophages with high cholesterol levels had increased TLR4 and MyD88/TRIF expression. HDLs also inhibit NF-κB–dependent responses to TLR ligands in an ABCA1-dependent manner, possibly by decreasing the cholesterol content of lipid rafts and reducing MyD88-dependent TLR trafficking to these domains. 34 This observation is consistent with the finding that the anti-inflammatory effects of HDLs and apolipoprotein A-I are enhanced in cholesterol-depleted macrophages and monocytes. 35,36 When these findings are considered in light of the current results, which establish that apolipoprotein A-I inhibits PG-PS–induced inflammation in HMDMs in an ABCA1-dependent manner, it follows that the observed reduction in the inflammatory response in the HMDMs in the present study may have been related to a reduction in the cholesterol content of the cells. Although it could be argued that a reduction in cell cholesterol levels cannot explain why CSL-111 inhibited inflammation in HMDMs in an ABCA1-dependent manner, it is noteworthy that this result is in agreement with a recent report in which the ability of the closely related formulation, CSL-112, to efflux cholesterol from macrophages was shown to be dependent on ABCA1. 37 Although the possibility of carrying out bone marrow transplantation studies to test this possibility in vivo is appealing, such an approach is unlikely to provide a definitive answer. The confounding issue in experiments of this type is that the infused apolipoprotein A-I and CSL-111 would incorporate rapidly into the endogenous HDL fraction, where they would be remodeled by plasma factors. 38,39 This would result in the generation of several subpopulations of HDL particles with the capacity to accept cellular cholesterol by multiple pathways.

In conclusion, this study provides mechanistic insights into the potential therapeutic benefit of apolipoprotein A-I and HDL infusions in arthritis. The results also suggest that HDL- and apolipoprotein A-I–raising agents that are currently being investigated in large-scale cardiovascular clinical outcome trials may be useful in other inflammatory disorders that can be refractory to conventional treatments.

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Disclosures

None.

References


**Significance**

The ability of high-density lipoproteins (HDLs) and the main HDL apolipoprotein A-I to inhibit vascular inflammation is well established. The present study shows that the anti-inflammatory properties of HDLs and apolipoprotein A-I extend beyond the vasculature to the inhibition of joint inflammation and joint destruction in an animal model of arthritis. These results were associated with reduced accumulation of inflammatory cells, as well as decreased expression of proinflammatory cytokines, toll-like receptor 2, and the master regulator of inflammation, nuclear factor-κB, in the joints. In vitro studies established that these effects of apolipoprotein A-I and HDLs were dependent on the ATP-binding cassette transporters A1 and G1 that export cholesterol from cells. When taken together, these results suggest that the HDL- and apolipoprotein A-I–raising agents currently being investigated in large-scale clinical cardiovascular outcome trials may also be beneficial for people with rheumatoid arthritis, especially those are refractory to, or unable to tolerate, conventional therapies.
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MATERIALS AND METHODS

Inhibition of Arthritis in the Lewis Rat by Apolipoprotein A-I and Reconstituted High Density Lipoproteins

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Materials and Methods

Animal studies

Arthritis was induced in five groups of female Lewis rats (Groups 2, 3, 4 and 5, n=6-7/group, approximate weight 150 g, Animal Resources Centre, Perth, Australia) with a single intraperitoneal (ip) injection of peptidoglycan-polysaccharide (PG-PS, 15 mg/kg body weight, BD Pharmingen, Australia, Catalogue Number: 210866) (Supplemental Fig. I). Control animals (n=7/group) received an equivalent volume of ip saline (Group 1).

The animals in Groups 1 (n=7/group) and 2 (n=7/group) received an intravenous (iv) saline infusion 1 day prior to and on days 1, 7, 9 and 11 after the ip saline or PG-PS administration (Fig. 1). The animals were treated with lipid-free apolipoprotein (apo) A-I as follows: (Group 3) two iv infusions of apoA-I (8 mg/kg) 24 h before and 24 h after the ip PG-PS injection, followed by three iv saline infusions on days 7, 9 and 11 after the PG-PS injection (n=6/group), (Group 4) two iv infusions of saline 24 h before and 24 h after the ip PG-PS injection, followed by three iv apoA-I (8 mg/kg) infusions on days 7, 9 and 11 post-PG-PS injection (n=6/group), and (Group 5) two iv infusions of apoA-I (8 mg/kg) 24 h before and 24 h after the ip PG-PS injection and three iv apoA-I infusions (8 mg/kg) on days 7, 9 and 11 post-PG-PS injection (n=3/group) (Supplemental Fig. I). The iv infusions of saline and apoA-I were administered via a tail vein.

Joint inflammation was assessed by two blinded observers and quantified as a combined forepaw and hindpaw score using a standard joint inflammation score:\(^1\): 0–no inflammation, 1–slight redness and swelling of the foot, 2–swelling of the foot such that the tendons are no longer visible, 3–swelling extending to the ankle joint and 4–gross inflammation and deformity of the ankle joint.

The ability of HDL to inhibit joint inflammation was investigated in an additional two groups of female Lewis rats (n=6/group) that received a single ip injection of PG-PS (15 mg/kg) and iv infusions of saline or a preparation of reconstituted HDL consisting of apoA-I (8 mg/kg) complexed with soybean phosphatidylcholine 24 h prior to and at days 1, 7, 9, 11 after the PG-PS injection. The reconstituted HDL preparation (CSL-111) was donated by CSL, Ltd, Parkville, Australia.

All the animals were sacrificed on day 21 after ip PG-PS or saline administration. Blood was obtained at sacrifice and plasma isolated by centrifugation. Synovial lavage fluids were collected after an intra-articular injection of saline (200 µL) into the ankle. The right hind ankles were removed and fixed in 4% (v/v) formaldehyde for immunohistochemical assessment. Synovial tissues were isolated from the left hind ankles. All of the procedures were approved by the Sydney Local Health Network Animal Welfare Committee (Approval Number 2008/011).

Immunohistochemistry

Tibiotalar joints were fixed for 4 days in formaldehyde, then decalcified in 0.3 M/L EDTA-Na\(_2\) (pH 7.0) for 4 weeks\(^5\). The joints were dehydrated with 70% (v/v) alcohol and embedded in paraffin. Longitudinal sections (5 µm) were incubated overnight at 4 °C with a mouse anti-rat CD68 monoclonal antibody (1:100) (AbD Serotec, Raleigh, NC, Catalogue Number: MCA341R), treated with Horse Radish Peroxidase (HRP)-3,3’ Diaminobenzidine (DAB) (Envision Mouse Kit, DAKO, Glostrup, Denmark, Catalogue Number: K4007), and counter stained with haematoxylin. The sections were imaged using an upright light microscope (Zeiss, Jena, Germany) at 50x magnification. DAB staining was quantified with ImageJ software (http://rsb.info.nih.gov/ij/). The threshold for positive staining was defined by an independent observer that was blinded to the treatment. Data are expressed as the intensity (pixels) of CD68 positive staining.

White blood cell count and quantification of cytokine levels

White blood cell (WBC), neutrophil and monocyte counts, were determined as described\(^3\). Briefly, total WBCs were manually counted using a hemocytometer. Neutrophils and...
monocyte numbers were determined on blood smears treated with Diff Quik Stain (Lab. Aids, Australia).

Total synovial fluid WBCs, neutrophils and monocytes were manually counted at a high magnification (40x objective) using synovial fluid smears prepared with a Cytospin 4 cytocentrifuge (Thermo Scientific Cytospin 4, Kalamazoo, MI) and treated with Diff Quik Stain. Plasma and synovial fluid TNF-α, IL-1β and IL-6 levels were determined by ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, Catalogue Number: DY510, DY501 and DY506, respectively).

**Flow cytometry**

For WBC immuno fluorescence staining, whole blood was treated with lysing buffer (Gibco by Life Technologies, Australia) to remove red blood cells. The samples were then incubated on ice for 30 min with FITC mouse anti-rat CD44 and/or PE mouse anti-rat CD11b/c antibodies (BD Pharmingen, Australia, Catalogue Number: 550974 and 554862, respectively), washed with PBS, fixed in 1% (v/v) paraformaldehyde and analyzed using a FACSVantage flow cytometer (BD Biosciences, Franklin Lakes, NJ). Data was analyzed with Cell Quest software. Typically, 10,000 cells were analyzed based on forward versus side scatter gating.

**Preparation of lipid-free apoA-I**

High density lipoproteins (HDL) were isolated from pooled samples of normal human plasma (Healthscope Pathology, South Australia) by sequential ultracentrifugation (1.063<d<1.21 g/ml). The HDL were delipidated and apoA-I was isolated by chromatography on a Q Sepharose Fast Flow column attached to an Äkta–FPLC system (GE Healthcare, Chalfont St Giles, Bucks, UK), washed with PBS, fixed in 1% (v/v) paraformaldehyde and analyzed using a FACSVantage flow cytometer (BD Biosciences, Franklin Lakes, NJ). The p65 subunit of nuclear factor (NF) κB was quantified by western blotting. TNF-α, IL-1β and IL-6 levels in the culture media were determined by ELISA according to the manufacturer’s recommendations (R&D Systems, Minneapolis, MN).

**Cell Culture and transfections**

Human monocyte-derived macrophages (HMDMs) were prepared using white cell buffy coats obtained from healthy donors (Australian Red Cross Blood Services, Sydney, Australia) in a Beckman Avanti J-20 XPI centrifuge as previously described, then differentiated at 37 °C in RPMI (Roswell Park Memorial Institute) 1640 medium containing heat-inactivated human serum supplemented with L-glutamine (20 mM/L), penicillin (100 i.u./mL) and streptomycin (100 µg/mL) and incubated for 6 h. The HDL were delipidated and apoA-I (final concentration 0.25, 0.5 and 1.0 mg/mL) or CSL (final apoA-I concentration 1.0 mg/mL) was added to the cells, which were then stimulated for 6 h with PG-PS (final concentration 20 µg/mL). TLR2 and MyD88 mRNA levels were quantified by qPCR. The p65 subunit of nuclear factor (NF)-κB was quantified by western blotting. TNF-α, IL-1β and IL-6 levels in the culture media were determined by ELISA according to the manufacturer’s recommendations (R&D Systems, Minneapolis, MN).

TLR2 was silenced by transfecting HMDMs at 37 °C for 24 h with specific TLR2 siRNAs (Santa-Cruz Biotechnology, Catalogue Number: sc-40256) or scrambled siRNA (control) (Santa-Cruz Biotechnology, Catalogue Number: sc-37007). ABCA1, ABCG1 and SR-B1 were silenced by transfecting HMDMs at 37 °C for 48 h with specific ABCA1, ABCG1 and SR-B1 siRNAs (200 pmol, SMARTpool, mixture of four different target-specific sequences,) or scrambled siRNA (control) (Thermo Scientific, Lafayette, CO, Catalogue Number: L-008615-00-005, L-004128-00-0005, L-010592-00-0005 and D-001810-10-15, respectively) using the Opti-MEM/Lipofectamine system (Invitrogen, Carlsbad, CA, Catalogue Number: 772661).
**Western Blotting**

Nuclear proteins from HMDMs were isolated using the NE-PER extraction kit (Pierce, Rockford, IL, Catalogue Number: 78833), and lysed in 20 mM/L Tris buffer (pH 7.5) containing 0.5 mM/L EDTA-Na₂, 0.5 mM/L EGTA-Na₂ and protease inhibitors. The nuclear lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, Catalogue Number: NP0321) and incubated overnight with an NF-κB p65 rabbit anti-human polyclonal antibody (1:500) (Santa-Cruz Biotechnology, Catalogue Number: sc-372), or TLR2 mouse anti-human (1:200) (Santa-Cruz Biotechnology, Catalogue Number: sc-166900), ABCA1 mouse anti-human (1:500) (Abcam, Catalogue Number: ab18180), ABCG1 rabbit anti-human (1:500) (Abcam, Catalogue Number: ab52617), or SR-B1 rabbit anti-human (1:200) (Epitomics, Catalogue Number: 1971-1) monoclonal antibodies using a mouse anti-human monoclonal antibody against β-actin (1:3000) (Sigma-Aldrich, Catalogue Number: A1978) as a loading control. Anti-rabbit and anti-mouse IgG-HRP (Santa-Cruz Biotechnology, Catalogue Number: sc-2004 and sc-2005, respectively) were used as secondary antibodies. Immunoreactive proteins were detected by ECL and analysed with Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

**qPCR**

Synovial tissues were incubated in RNAlater solution (Ambion, Austin, TX, Catalogue Number: 7020) at 4 °C for 24 h, then stored at -80 °C until use. Total RNA was isolated from the frozen tissues using TRIzol (Invitrogen, Carlsbad, CA, Catalogue Number: 15596018), and from HCAECs using TRI reagent (Sigma-Aldrich, Catalogue Number: 93289). RNA concentrations were normalized to 100 ng/µL using the SYBR Green II assay (Molecular Probes, Invitrogen, Carlsbad, CA, Catalogue Number: S7568) and reverse transcribed using iSCRIPT/iQ SYBR Green Supermix in a BioRad iQ5 thermocycler. Relative changes in mRNA levels were determined by the ΔΔCT method, using β-actin as a control. The PCR primers were: rat β-actin: Sense 5'-GCCCTGGCTCCTAGCACC-3', Antisense 5'-CCACCAATCCACACAGTACTTG-3'; rat TNF-a: Sense 5'-TACTGAACTTCCGGGTGATTTGTC-3', Antisense: 5'-CAGCCTTGTCCCTGAAAGAACC-3'; rat TLR2: Sense 5'-CTCCTGTGAACTCCTGTCCCTT-3', Antisense: 5'-AGCTGTCTGGCCAGTCAAC-3'; human β-actin: Sense 5'-GATCGCTGACCGTATGCAG-3', Antisense 5'-GTCGTACTCTCCTGCTTGGT-3'; human TLR2: Sense 5'-GGCCAGCAAATTACCTGTGTG-3', Antisense 5'-AGGCCGACATCTGGACTG-3'; human MyD88: Sense 5'-GACCGTTTCTAGTCTGGCTC-3', Antisense 5'-GGATACCAATTACCTGTGTG-3'; human NFκB: Sense 5'-GGAGGGATCTGCTGGCTG-3', Antisense 5'-GGAAGGATCTGCTGGCTG-3'; human ABCA1: Sense 5'-GAGGTCTCCCGAGTTGGTGT-3', Antisense 5'-GTATAAAAGAAGCCCTCCAGCATC-3'; human ABCG1: Sense 5'-TGCAATCTTGTGCAATATTGA-3', Antisense 5'-CCAGCGACTGTCTCATCA-3'; human ABCB1: Sense 5'-GGTCCCTGTCATCTGCCCC-3', Antisense 5'-CTCCTATCTTGTGCAATATTGA-3'.

**Statistics**

Data are expressed as the mean±SEM. One-way ANOVA and the Newman-Keul’s post-hoc test were used to evaluate differences between groups. Statistical analyses were carried out using GraphPad Prism software Version 4.03 (GraphPad Software, Inc. San Diego, CA). A value of p<0.05 was considered significant. All the data fulfilled the criteria for normal distribution as determined using the Kolmogorov-Smirnov test (SPSS Software v21).
References
SUPPLEMENTAL MATERIAL

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Supplemental Fig. I. Schematic representation of the study design for the prevention of experimental arthritis in female Lewis rats by apoA-I. ip, intraperitoneal injection; iv, intravenous infusion.
Supplemental Fig. II. ApoA-I inhibits the PG-PS-mediated activation of HMDMs in a TLR2-dependent manner. HMDMs were transfected with scrambled siRNA (siControl [siCtrl], open bars) or TLR2 siRNA (siTLR2) (closed bars). Transfection efficiency was monitored by western blotting with anti-TLR2 antibodies using β-actin as a loading control (Panel A), and by TLR2 mRNA levels (Panel B). Transfected HMDMs were then incubated with PBS or apoA-I (final concentration 1 mg/mL) for 16 h, prior to stimulation for a further 6 h in the absence or presence of PG-PS (final concentration 20 µg/mL). TLR2 mRNA levels were quantified by qPCR (Panel C). TNF-α levels in the culture medium were determined by ELISA (Panel D). Data represent mean±SEM (n=3). N.D = below the level of detection. *p<0.05 vs relative HMDMs transfected with scrambled siRNA. #p<0.05 vs PG-PS-treated cells incubated with PBS.
Supplemental Fig. III. Transfection of HMDMs with ABCA1 siRNA, ABCG1 siRNA and SR-B1 siRNA: Effects on protein and mRNA levels. HMDMs were transfected with scrambled siRNA (siCtrl, open bars), ABCA1 siRNA (siABCA1), ABCG1 siRNA (siABCG1), or SR-B1 siRNA (siSR-B1) (closed bars). Transfection efficiency was monitored by western blotting with anti-ABCA1 (Panel A), ABCG1 (Panel B) and SR-B1 (Panel C) antibodies at 24, 48 and 72 h using β-actin as a loading control. ABCA1, ABCG1 and SR-B1 mRNA levels were quantified at 48 h (Panels D, E and F, respectively). Data represent the mean±SEM (n=3 for all conditions except for HMDMs transfected with ABCA1 siRNA, where n=2). *p<0.05 vs scrambled siRNA.
Supplemental Fig IV. CSL-111 inhibits PG-PS-induced TLR2 expression and NF-κB activation in HMDMs. HMDMs were pre-incubated for 16 h with PBS or CSL-111 (final apoA-I concentration 1.0 mg/mL), then incubated for a further 6 h in the absence (open bars) or presence of PG-PS (final concentration 20 µg/mL, closed bars). TLR2 (Panel A) and MyD88 (Panel B) mRNA levels were quantified by qPCR. Nuclear fractions of cell lysates were subjected to SDS-PAGE and Western blotting with an anti-NF-κB p65 subunit (p65) antibody using β-actin as a loading control. Data are expressed as the intensity of the p65 band relative to β-actin (Panel C). TNF-α (Panel D), IL-6 (Panel E) and IL-1β (Panel F) levels in the culture medium were determined by ELISA. Data represent mean±SEM of 3-6 independent experiments. *p<0.05 vs PG-PS-treated cells incubated with PBS.
Supplemental Fig. V. CSL-111 inhibits the PG-PS-mediated activation of HMDMs in an ABCA1- and ABCG1-dependent manner. TLR2 mRNA levels were determined in HMDMs transfected with scrambled siRNA (siControl, open bars), ABCA1 siRNA (siABCA1), ABCG1 siRNA (siABCG1), or SR-B1 siRNA (siSR-B1) (closed bars), then incubated with PBS or apoA-I (final concentration 1 mg/mL) for 16 h prior to stimulation for a further 6 h in the absence or presence of PG-PS (final concentration 20 µg/mL). Data represent mean±SEM of 3-6 independent experiments. *p<0.05 vs PG-PS-treated cells treated incubated with PBS.
Supplemental Fig VI. CSL-111 protects against PG-PS-induced arthritis in female Lewis rats. Female Lewis rats received a single ip injection of PG-PS (15 mg/kg) and iv injections of saline or CSL-111 (8 mg/kg apoA-I) 24 h prior to and at days 1, 7, 9, 11 after the PG-PS injection. Pictures and quantification of joint inflammation 21 days after the ip PG-PS or saline injection and iv infusion of saline (n=6, closed circles) or CSL-111 (n=6, open circles) are shown. Arthritis scores were determined as described in Materials and Methods. Data are expressed as mean±SEM. *p<0.05 vs PG-PS treated, saline infused rats.