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Abstract
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EXTENDED REPORT

High-density lipoproteins inhibit urate crystal-induced inflammation in mice

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ABSTRACT

Objectives To investigate the effects and mechanisms of action of high-density lipoproteins (HDL) in monosodium urate (MSU) crystal-induced inflammation—that is, gouty inflammation, in vivo.

Methods Air pouches raised on the backs of mice were injected with MSU crystals or tumour necrosis factor (TNF) in the presence or absence of HDL and/or interleukin (IL)-1 receptor antagonist (IL-1Ra) for 3 h. Leucocyte count and neutrophil percentage in pouch fluids were measured using a haemocytometer and May–Grünwald–Giemsa staining. The cytokine production and expression in the pouch were measured by ELISA and quantitative RT-PCR.

Results MSU crystals induced leucocyte infiltration, mostly neutrophils, and the release of IL-1β, IL-6, chemokine (C-C motif) ligand 1 (CXCL1), chemokine (C-C motif) ligand 2 (CCL2) and IL-1Ra in pouch fluids. TNF remained under the detection limit. MSU crystals triggered IL-1β, IL-6 and CXCL1 expression in both pouch exudates and membranes, whereas CCL2 and TNF mRNA were not modified. The co-injection of MSU crystals and HDL inhibited leucocyte influx by 59% and neutrophil infiltration by 83% and, in turn, both protein and mRNA levels of all assessed proinflammatory cytokines were reduced, but not those of IL-1Ra. Similar results were obtained when mice were injected with MSU crystals pretreated with HDL or TNF instead of crystals. When HDL and IL-1Ra were added together they displayed additional inhibition, suggesting different mechanisms of action.

Conclusions This study demonstrated that HDL may represent an important factor in the modulation of gouty inflammation by acting on both tissue and infiltrating cells—that is, synovial tissue and synovial fluid cells. HDL display anti-inflammatory activity, in part, by interacting with crystals but also by directly acting on cells.

INTRODUCTION

The deposition of monosodium urate (MSU) crystals in joints induces an inflammatory response characterised by a cellular infiltrate rich in neutrophils and the production of inflammatory mediators.1 Importantly, a pivotal role of IL-1β and, in turn, of NALP3 inflammasome in this process has been indicated2 and confirmed by treating gouty patients with the IL-1 receptor antagonist (IL-1Ra, anakinra), which resulted in an improvement of 50–100% in all treated patients within <3 days.3

One of the characteristic features of crystal-induced inflammation is that acute attacks are self-limiting even without treatment. Many factors are involved in this spontaneous resolution.4–6 Among them, synovial fluid plasma proteins and lipoproteins have been shown to suppress the inflammatory response to MSU crystals.9 10 In particular, apolipoprotein (apo) B and apo E inhibit crystal-induced neutrophil stimulation by binding to the surface of crystals.11 12 Recently, we showed that high-density lipoproteins (HDL) might contribute to the resolution of acute gout attack by reducing chemokine (C-C motif) ligand 2 (CCL2) production in human synoviocytes, and inhibiting monocyte/macrophage recruitment in joints.13 Furthermore, preliminary results in the mouse subcutaneous air pouch model showed that HDL treatment decreased MSU crystal-induced release of interleukin (IL)-6, chemokine (C-X-C motif) ligand 1 (CXCL1) and CCL2 24 h after MSU injection.14 It was also shown that an atherogenic lipid profile with low serum HDL cholesterol level is an independent predictor for flares in gouty patients.15 HDL have an important role in reverse cholesterol transport and their anti-atherogenic and anti-inflammatory role has been widely described. The mechanisms of HDL anti-inflammatory effects have been only partially characterised. For instance, it is likely that HDL impede the interaction between monocytes and stimulated T cells or stimulated T cell-derived microparticles.16–18 In crystal-induced inflammation HDL may attenuate inflammatory activity by adhesion to crystals and/or by acting directly on cells through blockade of putative crystal receptors/sensors or diminution of the threshold of cell response to crystals.

The mouse air pouch model of local inflammation is particularly useful in the study of the basic mechanisms of arthritis and acute inflammatory responses. The injection of MSU crystals into the pouch induces a transient neutrophilic infiltration similar to that occurring in humans during an acute attack of gout and promotes the release of inflammatory mediators including cytokines such as IL-1β and IL-6, and chemokines such as CCL2 (MCP-1) and CXCL1 (KC), involved in monocyte/macrophage and polymorphonuclear cell (PMN) recruitment, respectively. Using this model in our study, we investigated the potential anti-inflammatory effects of HDL in crystal-induced inflammation in vivo.

MATERIALS AND METHODS

Animals

Male CD1 mice were maintained on a standard laboratory diet and received water ad libitum at the Experimental Surgery Center of Padova
University. All animals used for the experiments were aged 8–9 weeks and weighed 30–35 g.

Preparation of MSU crystals
MSU crystals were prepared as previously described and were determined to be free from endotoxin by Limulus amebocyte lysate assay (Sigma-Aldrich).

HDL isolation
Human serum HDL were isolated, and their protein content quantified, as previously described.

Air pouches
Air pouches were raised on the backs of the mice as previously described. MSU crystals in 1 mL of sterile, endotoxin-free phosphate-buffered saline (PBS) were injected into the pouches with or without HDL or human serum albumin (HSA, Calbiochem) (0.1 mg/mL). In some experiments MSU crystals were pretreated for 1 h with HDL or HSA (0.1 mg/mL) and recovered by centrifugation before injection into the subcutaneous space. Alternatively, 1 mL of murine tumour necrosis factor (TNF; 1.5 ng/mL) with or without HDL (0.1 mg/mL) was injected into the pouches. Controls received the same volume of sterile PBS alone or HDL or HSA (0.1 mg/mL) in PBS. Inhibition experiments were performed with 0.2 mg/mL of IL-1 receptor antagonist (IL-1Ra; R&D Systems) in the presence or absence of HDL (0.1 mg/mL). Seven mice per group were used. Mice were killed by CO₂ exposure and pouch fluids were harvested at specific time points by washing with 2 mL of PBS.

Collection and processing of samples
Immediately after collection, the exudates were assessed for total (haemocytometer) and differential (May–Grünewald–Giemsa staining) white blood cell (WBC) count. The exudates were then centrifuged at 1500 rpm for 10 min at 20°C (ALC PK 130 centrifuge, rotor No T535) and supernatants were kept at −80°C until cytokine/chemokine measurements were made, while the cell pellet was lysed in TRI reagent (Sigma-Aldrich) and kept at −80°C until RNA extraction. Air pouch membranes were carefully dissected from adjacent subcutaneous and paraspinal tissues. The isolated membranes were homogenised in TRI reagent immediately after dissection and stored at −80°C until further use.

Cytokine measurement
Cytokine concentration was measured in the pouch exudates by a commercially available enzyme immunoassay: IL-6, CCL2, TNF (BioLegend), CXCL1 (Invitrogen Corp), IL-1β (eBioscience) and IL-1Ra (RayBiotech).

Quantitative real-time PCR
Quantitative real-time duplex PCR analysis (TaqMan 7300 quantitative real-time PCR system, Applied Biosystems) was

Figure 1 Time course of cellular infiltration and cytokine levels in air pouch exudates in monosodium urate (MSU) crystal-induced inflammation in mice. MSU crystals (2 mg/mL) were injected into air pouches and the mice were killed after increasing periods of the time. The number of leucocytes (white blood cells (WBC)) (A) and the percentage of polymorphonuclear cells (PMN) (B) accumulated in air pouch wash fluids were determined. Supernatants from the air pouch wash fluids were analysed for IL-1β (C), IL-6 (D), CXCL1 (E) and CCL2 (F) by ELISA. Results are presented as mean±SD of seven mice per group. CCL2, chemokine (C-C motif) ligand 2; CXCL1, chemokine (C-X-C motif) ligand 1; IL, interleukin.
conducted after reverse transcription by SuperScript II (Invitrogen), as previously described. The levels of mRNA expression were normalised with the expression of a housekeeping gene (18S) analysed simultaneously (IL-1β, IL-6, CCL2, TNF, CXCL1 and 18S probes were purchased from Applied Biosystems). All measurements were conducted in triplicate.

Statistical analysis
Data are reported as mean±SD of seven experimental animals. Statistical differences between experimental groups were assessed by one-way analysis of variance followed by Dunn’s test. A p value <0.05 was taken as significant.

RESULTS
MSU crystals induce cell infiltration and cytokine production in the air pouch model
In preliminary experiments we determined that injection of MSU crystals into the subcutaneous pouches of mice induced a dose-dependent leucocyte accumulation and cytokine production, with a maximal effect observed at 2 mg/mL (data not shown). To verify the time points of peak and natural resolution of inflammation in the pouch, a time course experiment was performed, during which the leucocyte recruitment peaked 3 h after injection of 2 mg/mL MSU crystals (figure 1A), and at the same time PMN were the predominant cell type of the pouch exudates (figure 1B). Concomitantly with cell infiltration, IL-1β, IL-6, CXCL1 and CCL2 reached a maximum level 3 h after injection (figure 1C–F), while TNF levels remained under the detection limit (<8 pg/mL) in all pouch fluid supernatant samples (data not shown). Together these results show that inflammation induced by MSU crystals reached a maximum at 3 h with a diminution of both cell infiltrate and cytokine production after 6 h. Interestingly, total cell and PMN numbers as well as CXCL1 levels increased again 24 h after the injection of crystals (figure 1A,B,E), whereas other cytokines remained at basal level.

Figure 2
High-density lipoproteins (HDL) modulate cell infiltration and cytokine production induced by monosodium urate (MSU) crystals in mouse air pouch exudates. Dorsal air pouches raised on CD1 mice were injected (grey columns) or not (white columns) with 2 mg/mL of MSU crystals in the presence or absence of 0.1 mg/mL of HDL (MSU+HDL). Alternatively, MSU crystals were pretreated with HDL (0.1 mg/mL) and then injected into air pouches at a concentration of 2 mg/mL (HDL+MSU). Mice were killed after 3 h and the number of leucocytes (white blood cells (WBC)) (A) and the percentage of polymorphonuclear cells (PMN) (B) accumulated in air pouch wash fluids were determined. Supernatants from the air pouch wash fluids were analysed for IL-1β (C), IL-6 (D), CXCL1 (E), CCL2 (F) and IL-1Ra (G) by ELISA. Results are presented as mean±SD of seven mice per group. *p<0.05 vs PBS group, **p<0.01 vs PBS group, *p<0.05 vs MSU group, **p<0.01 vs MSU group. CCL2, chemokine (C-C motif) ligand 2; CXCL1, chemokine (C-X-C motif) ligand 1; IL, interleukin; IL1-Ra, interleukin 1 receptor antagonist; PBS, phosphate-buffered saline.

HDL modulate cell infiltration and cytokine production induced by MSU crystals

In a previous study, we showed that 0.1 mg/mL of HDL inhibit MSU crystal-induced CCL2 production in human synoviocytes. Therefore, MSU crystals were injected into the air pouches in the presence or absence of 0.1 mg/mL of HDL and the inflammatory response was evaluated after 3 h. As shown in figure 2, the co-injection of MSU crystals and HDL (MSU + HDL) significantly prevented leucocyte influx and PMN infiltration into the pouch exudates. Concomitantly, the secretion of all the proinflammatory cytokines assessed was inhibited, but not that of the anti-inflammatory cytokine IL-1Ra. Indeed, in the presence of HDL, the WBC and PMN accumulation was inhibited by 59% and 83%, respectively. In parallel, HDL reduced the release of IL-1β, IL-6, CXCL1 and CCL2 induced by MSU crystals by 11-, 3-, 3- and 2-fold, respectively, whereas the production of IL-1Ra, remained similar to that induced by crystals alone. Administration of the same volume of sterile PBS alone or HDL did not induce cell accumulation and cytokine production in pouches.

To assess whether this anti-inflammatory effect was due to the formation of complexes between HDL and MSU crystals, mice were injected with crystals pretreated with HDL (HDL + MSU) or with TNF in the presence or absence of HDL. Similarly, injection of MSU crystals pretreated with HDL prevented leucocyte influx into the pouches and the production and expression of the inflammatory factors tested, although in this latter setting, the inhibition of leucocyte and, in particular, PMN recruitment tended to be less pronounced than that seen after co-injection of MSU crystals and HDL (figure 2). Here again, the production of IL-Ra was not affected. TNF itself induced the recruitment of 6.58 ± 0.34×10^5 WBC and 61 ± 3.07% PMN into the pouch after 3 h. When HDL were administered, only 2.22 ± 0.12×10^5 WBC were present in the pouch exudates, representing 66% inhibition of the TNF-induced WBC infiltration. The PMN recruitment values dropped to an average of 16.5 ± 0.52% PMN/pouch, representing an inhibition of 73% (figure 3A,B). The injection of TNF increased the level of CXCL1 and CCL2 as compared with the administration of PBS alone. Treatment with HDL inhibited this TNF-dependent increase in protein levels for both factors (figure 3C,D). IL-1β and IL-6 remained below the detection limit in all pouch fluid supernatant samples (data not shown). Because TNF was the stimulus, TNF was not measured. Since it is unlikely that HDL directly interact with TNF, these results suggest that HDL affect cell responses.

To ascertain that the anti-inflammatory effect was specific to HDL, similar experiments were performed in the presence of HSA instead of HDL. As expected, HSA itself did not induce inflammation (see online supplementary figure S1). When pouches were injected with MSU crystals and HSA together or with MSU crystals pretreated with HSA, the cell counts and the release of proinflammatory cytokines remained similar to those induced by MSU crystals alone.

In spite of large variations between mice in the same group, IL-1β, IL-6 and CXCL1 mRNA expression was enhanced by MSU crystals in both pouch exudates (figure 4A) and membranes (figure 4B), whereas CCL2 and TNF mRNAs were not modulated. The co-injection of MSU crystals and HDL reduced the levels of CXCL1 and IL-6 mRNA in exudates, but not in membranes (figure 4A,B). IL-1β mRNA was not inhibited by HDL in exudates, whereas it was significantly diminished in membranes. When MSU crystals pretreated with HDL were injected into the pouches, IL-6 and CXCL1 mRNA expression in exudates was significantly inhibited, whereas only IL-1β mRNA expression was significantly inhibited in membranes, although IL-6 mRNA tended to be diminished without reaching significance (p=0.116). Finally, CCL2 mRNA expression in exudates was significantly diminished in all conditions with HDL, suggesting that the basal levels were already slightly enhanced by air and PBS injections (figure 4A). These results suggest that HDL mainly displayed their inhibitory activity by directly acting on exudate cells. However, part of the HDL inhibitory activity might also be attributed to direct interaction between crystals and HDL when considering IL-6 and CXCL1 mRNA expression in pouch exudates and IL-1β mRNA expression by membrane resident cells.

**Figure 3** High-density lipoproteins (HDL) inhibit cell infiltration and cytokine production induced by tumour necrosis factor (TNF) in mouse air pouch exudates. Dorsal air pouches raised on CD1 mice were injected (black columns) or not (white columns) with 1.5 ng/mL of TNF in the presence or absence of 0.1 mg/mL of HDL (TNF + HDL). Mice were killed after 3 h and the number of leucocytes (white blood cells (WBC)) (A) and the percentage of polymorphonuclear cells (PMN) (B) accumulated in air pouch wash fluids were determined. Supernatants from the air pouch wash fluids were analysed for CXCL1 (C) and CCL2 (D) by ELISA. Results are presented as mean±SD of seven mice per group. *p<0.05 vs PBS group, #p<0.01 vs PBS group, **p<0.005 vs TNF group, ***p<0.001 vs TNF group. CCL2, chemokine (C-C motif) ligand 2; CXCL1, chemokine (C-X-C motif) ligand 1; PBS, phosphate-buffered saline.
Together these results demonstrate that treatment with HDL decreases MSU-induced inflammation in this model by reducing WBC and PMN migration into the pouch fluid and by reducing the production of proinflammatory factors without affecting that of anti-inflammatory IL-1Ra.

**HDL directly inhibit the proinflammatory cytokine production induced by MSU crystals**

To determine whether IL-1 blockade might play a part in the reduction in inflammatory response in the air pouch model, the effect of the co-administration of MSU crystals and IL-1Ra (MSU+IL-1Ra) was assessed. The injection of IL-1Ra together with MSU crystals inhibited by 70% WBC count and 76% PMN recruitment induced by MSU crystals alone (figure 3A,B). Moreover, IL-1Ra caused a significant reduction in IL-6 and CXCL1 levels (figure 3D,E) but failed to inhibit the release of IL-1β and CCL2 (figure 3C,F). These data suggest that IL-6 and CXCL1 secretion and the related massive leucocyte and PMN recruitment might depend in part on IL-1β release induced by MSU crystals. In contrast, the production of IL-1β and CCL2 was directly induced by MSU crystals, ruling out the participation of a putative autocrine loop of IL-1. IL-1β and CCL2 release was similarly reduced in the presence of HDL (MSU+HDL and MSU+HDL+IL-1Ra), suggesting that HDL directly inhibited IL-1β and CCL2 induction by MSU, and that the inhibition of IL-1β production by HDL was, in turn, responsible for a large part of the diminution of IL-6 and CXCL1 production.

**DISCUSSION**

In this study, we demonstrate that HDL inhibit MSU-induced inflammation in the murine air pouch model. These results, obtained in vivo, confirm previous in vitro data demonstrating the anti-inflammatory effects of HDL in MSU-activated cells.

The injection of urate crystals causes a leucocyte influx and a rapid increase of IL-1β, IL-6, CCL2 and CXCL1 in pouch exudates. After 3 h, MSU crystals induce IL-1β, IL-6 and CXCL1 gene transcription in both pouch exudates and membranes, whereas CCL2 mRNA is not modulated. This might be because longer periods could be required to trigger CCL2 mRNA transcription, CCL2 being stocked in small cytoplasmic vesicles, and prone to be secreted upon stimulation.13
HDL administration considerably inhibits MSU crystal-induced inflammation. Reduction of the WBC and PMN counts in the pouch exudates of mice treated with HDL parallels the reduction of levels of all proinflammatory mediators tested but not those of the anti-inflammatory cytokine IL-1Ra. Noticeably, the levels of IL-1Ra production were around 10-fold higher than those of IL-1β—that is, not sufficient to inhibit the inflammatory effects of IL-1β, which requires 100–1000-fold excess concentration of IL-1Ra over IL-1. These results extend the preliminary data obtained in vivo by showing that the anti-inflammatory effect of HDL is not only evident in the late phase of crystal-induced acute arthritis (24 h), but as soon as 3 h after the injection of MSU crystals, when the inflammation reaches its highest level.

In addition, our results demonstrate that HDL display anti-inflammatory activity by affecting pro- and anti-inflammatory cytokine balance. This is in agreement with previous reports demonstrating that HDL display anti-inflammatory properties in both in vitro and in vivo experimental models and that lipoproteins may modulate crystal-induced inflammation. However, the mechanisms of HDL anti-inflammatory effects are only partially identified. It was shown that HDL and apo-AI prevent the release of inflammatory cytokines and radical oxygen species, by blocking the interaction of T cell ligands with the monocytes/macrophages and neutrophils. Furthermore, other studies have demonstrated the effectiveness of HDL and apo-AI in directly preventing the activation of neutrophils and monocytes. We recently showed that HDL reduce the production and expression of CCL2 in synoviocytes stimulated with MSU crystals by acting on resident fibroblast-like synoviocytes, offering an attractive explanation for clinical observations such as the self-limiting nature of the acute attack and striking variability in the inflammatory response to urate crystals. Conversely, in other studies, lipoproteins are suspected to attenuate the inflammatory activity of MSU crystals by directly interacting with the crystals. In this study, the inhibitory activity of HDL on crystal-induced inflammation is maintained even after administration of MSU crystals.
preincubated with lipoproteins into air pouch. On the other hand, similar modulatory effects on the leucocyte influx and the production of inflammatory factors were seen when HDL were co-injected with another stimulus such as TNF, suggesting a direct effect of HDL on cells.

The inhibition of MSU crystal-induced inflammation is specific to HDL since no effect was seen with HSA, a protein that binds MSU crystals.29 Thus the specific anti-inflammatory activity of HDL could be explained by the adhesion of lipoproteins to MSU crystals, and also by their direct interaction with inflammatory cells. This is further supported by the observation that treatment with HDL also reduces CCL2 mRNA expression induced by air and PBS injections at the basal levels. HDL decrease mRNA levels of all inflammatory factors tested in pouch exudates, and the mRNA levels of IL-1β and IL-6 in membranes, suggesting that they exert anti-inflammatory properties by acting on both tissue and infiltrating cells. By analogy, it is likely that HDL act on both synovial tissue and synovial fluid cells of gouty joints. mRNA levels of CXCL1 in membranes were not affected by the HDL treatment, possibly owing to the presence of different cell types in the membranes compared with exudates. The high mRNA levels of CXCL1 may explain the increased CXCL1 concentration in the exudates, which is observed 24 h after crystal injection into the pouch (see figure 1). This, in turn, could be the cause of the large number of leucocytes, in particular PMN, seen after 24 h.

IL-1β is thought to be a key cytokine in MSU crystal-induced inflammation.2,3 We observed that the administration of IL-1Ra/ankintra together with MSU crystals into the pouches reduces the number of inflammatory cells, as compared with administration of MSU crystals alone. IL-1Ra also reduces IL-6 and CXCL1 levels in the exudates but does not affect IL-1β and CCL2 release, suggesting that the production of IL-6 and CXCL1 induced by MSU crystals is, in a large part, mediated by an autocrine loop of IL-1. This confirms previous in vitro results showing that MSU crystals directly induce IL-1β and CCL2 release through activation of NALP3 inflammasome and degranulation of intracellular vesicles, respectively.2 13 In addition, here we show that HDL display anti-inflammatory properties in MSU crystal-induced inflammation by decreasing the production and expression of inflammatory factors in mice, thus suggesting that HDL may directly reduce the major proinflammatory cytokines involved in gout.

In conclusion, this study demonstrates that HDL modify the local inflammatory response induced by MSU crystals in vivo decreasing the recruitment of leucocytes, in particular of PMN, and reducing the production and expression of inflammatory mediators, without affecting the production of anti-inflammatory factors such as IL-1Ra. The mechanisms of action behind the effects of HDL include the direct interaction of HDL with MSU crystals and the interaction of lipoproteins with both infiltrating cells and pouch tissue cells. This study strengthens the importance of HDL as a modulator of gouty inflammation. Further studies should elucidate the precise mechanism by which HDL exert this anti-inflammatory effect.

**Contributors** AS designed the study, performed the experiments, analysed the data, drafted and revised the paper. RL performed the experiments and analysed the data. FO, PS participated in the design of the study, analysed the data, drafted and revised the paper. LP designed the study, drafted and revised the paper.

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**Competing interests** None.

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