High-density lipoprotein prevents SAA-induced production of TNF-α in THP-1 monocytic cells and peripheral blood mononuclear cells

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In this study, we evaluated whether human serum and lipoproteins, especially high-density lipoprotein (HDL), affected serum amyloid A (SAA)-induced cytokine release. We verified the effects of SAA on THP-1 cells in serum-free medium compared to medium containing human serum or lipoprotein-deficient serum. SAA-induced tumour necrosis factor-alpha (TNF-α) production was higher in the medium containing lipoprotein-deficient serum than in the medium containing normal human serum. The addition of HDL inhibited the SAA-induced TNF-α release in a dose-dependent manner. This inhibitory effect was specific for HDL and was not affected by low-density lipoprotein or very low-density lipoprotein. In human peripheral blood mononuclear cells, the inhibitory effect of HDL on TNF-α production induced by SAA was less pronounced. However, this effect was significant when HDL was added to lipoprotein-deficient medium. In addition, a similar inhibitory effect was observed for interleukin-1 beta release. These findings confirm the important role of HDL and support our previous hypothesis that HDL inhibits the effects of SAA during SAA transport in the bloodstream. Moreover, the HDL-induced reduction in the proinflammatory activity of SAA emphasizes the involvement of SAA in diseases, such as atherosclerosis, that are characterized by low levels of HDL.

Key words: human serum - lipoprotein-deficient serum - peripheral blood mononuclear cells - proinflammatory cytokines - serum amyloid A - THP-1 monocytic lineage

High-density lipoprotein (HDL) particles are multifunctional lipoprotein complexes that transport lipids and they have anti-inflammatory properties that are associated with protection against atherosclerosis and other inflammatory diseases (Barter et al. 2004, Wu et al. 2004). The anti-inflammatory properties of HDL include the inhibition of adhesion molecule expression (Cockerill et al. 1995), the stimulation of endothelial nitric oxide synthase (eNOS) production (Yuhanna et al. 2001) and the protection of low-density lipoprotein (LDL) against peroxidative damage (Mackness et al. 2000). HDL and other plasma lipoproteins can also bind and neutralise the activity of Gram-negative bacterial lipopolysaccharide (LPS) (Munford et al. 1982) and Gram-positive bacterial lipoteichoic acid (Grunfeld et al. 1999), which elicit strong proinflammatory responses.

The knowledge regarding the biological roles of the acute-phase protein serum amyloid A (SAA) has greatly increased in the past few years. For example, its involvement in many human diseases, such as obesity and its metabolic complications (Yang et al. 2006) and chronic inflammatory diseases (Yamada et al. 1996, Gutfeld et al. 2006), is much better understood. In previous studies, we demonstrated the following immunomodulatory activities of SAA on human leukocytes: the induction of the expression and release of tumour necrosis factor-alpha (TNF-α) (Hatanaka et al. 2004), interleukin-1 beta (IL-1β) (Furlaneto & Campa 2000), interleukin-8 (IL-8) (Ribeiro et al. 2003) and CCL20 (Sandri et al. 2008a), the priming activity for opsonised particles (Hatanaka et al. 2003) and the production of nitric oxide (Sandri et al. 2008b). Recently, SAA was proposed to control the plasticity of neutrophil differentiation (De Santo et al. 2010).

The production of SAA is usually triggered by proinflammatory cytokines and SAA is mainly secreted by the liver (Uhlar & Whitehead 1999). SAA reaches the bloodstream and associates with HDL, thereby becoming the major HDL apolipoprotein during acute-phase immune responses (Malle & De Beer 1996). Many reports have described the extrahepatic production of SAA by various tissues and cells such as atherosclerotic lesions (Meek et al. 1994), synovial tissue (O'Hara et al. 2004), endothelial cells (Meek et al. 1994), activated macrophages (Urieli-Shoval et al. 1994) and adipocytes (Poitou et al. 2005). Delipidated SAA is expected to be present at the aforementioned sites.

The biological activities of SAA are dependent on its association with lipids. In vitro studies have described the inhibitory effect of HDL on SAA-mediated activities, including the induction of chemotaxis (Badolato et al. 1994), the release of tissue factor (Cai et al. 2007) and IL-8 (Baranova et al. 2010) and TNF-α mRNA expression (Song et al. 2009a, b). Previously, we demonstrated that the proinflammatory effects of SAA are related to the free form of SAA. HDL from acute-phase patients did not trigger the release of cytokines (Furlaneto & Campa 2000). In the current study, we extended our previous findings by showing that HDL prevented SAA-induced TNF-α production in THP-1 monocytic cells and peripheral blood mononuclear cells (PBMC).

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MATERIALS AND METHODS

Reagents - Histopaque®, Percoll and Roswell Park Memorial Institute (RPMI)-1640 that was supplemented with glutamine and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Foetal bovine serum (FBS), penicillin, phosphate-buffered saline (10 x) and streptomycin were acquired from Gibco (Grand Island, NY, USA). Recombinant human SAA was purchased from PeproTech Inc (Rocky Hill, NJ, USA) and the amount of endotoxin was lower than 0.1 ng/μg of protein according to the supplier. Human lipoprotein-deficient serum (LDS) was purchased from Sigma-Aldrich and the content of lipoprotein was less than 5% of normal according to the supplier.

Human serum - Human serum was obtained from total blood that was collected from healthy volunteers. The serum was separated by centrifugation at 1,500 *g* for 10 min at 4ºC and inactivated at 56ºC for 30 min. The blood collection was performed according to a protocol that was approved by the Ethical Committee of the School of Pharmaceutical Sciences at São Paulo University (CEP/FCF 465).

Cell preparation and culture - THP-1 cells (human monocytic cell lineage) were kindly provided by Dr Hugo Pequeno Monteiro (UNIFESP, Brazil) and were cultured in RPMI-1640 medium that was supplemented with FBS (10%), penicillin (100 IU/mL) and streptomycin (100 μg/mL). Human PBMC were isolated from fresh heparinised venous blood using density-gradient centrifugation and Histopaque® (d = 1.077) as previously described (Boyum 1974). For culturing, 2.0 x 10^6 cells/mL were resuspended in RPMI-1640 medium that was supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were plated into each well of a 96-well flat-bottom tissue-culture plate (Corning, Corning, NY, USA). The cultures were incubated at 37ºC in an atmosphere of 5% CO_2.

Stimulation of cells - Before the cells were stimulated, SAA (17 μg/mL) and different concentrations of lipoproteins [HDL, LDL and very LDL (VLDL)] were pre-incubated with serum-free medium (SFM) or medium that was supplemented with 10% human serum or lipoprotein-deficient serum at 37ºC for 30 min under mild shaking and subsequently added to the cell cultures.

Isolation of lipoproteins - The VLDL, LDL and HDL lipoproteins were isolated from a pool of plasma that was obtained from the venous blood of healthy volunteers and collected in tubes containing ethylenediamine tetraacetic acid (1 mg/mL) (Becton, Dickinson, Franklin Lakes NJ, USA). The plasma was immediately separated by centrifugation at 1,500 *g* for 10 min at 4ºC. VLDL (density, 1,006 g/mL), LDL (density, 1,065 g/mL) and HDL (density, 1.21 g/mL) were separated using a KBr solution and sequential flotation ultracentrifugation as previously described (Havel et al. 1955). The recovered lipoproteins were dialysed against cold 0.9% NaCl. The lipoproteins samples were concentrated using a Savant SC110 SpeedVac. The protein concentrations of the lipoprotein particles were determined using the Lowry method and bovine serum albumin as the standard (Lowry et al. 1951). The lipoprotein samples were filtered through sterile filters (Millipore®, 0.22 μm) and stored at 4ºC.

Measurement of cytokines - Cell-free supernatants were collected, centrifuged and assayed for TNF-α and IL-1β using the enzyme-linked immunosorbent assay (DuoSet, R&D Systems, Minneapolis, MN, USA) according to the protocol that was recommended by the supplier.

Statistical analysis - Statistical analyses were based on one-way analysis of variance followed a the Student-Newman-Keuls multiple comparisons test using Prism v5.0 software from GraphPad (San Diego, CA, USA) for comparison among SFM, LDS-containing medium and serum-containing medium and for the analysis of lipoprotein-mediated effects. A t-test was used to compare SAA-stimulated and unstimulated cells.

RESULTS

Effects of human serum and HDL on SAA-induced TNF-α production by THP-1 cells - The SAA-induced TNF-α release was strongly affected by the presence and type of serum (Fig. 1A). First, we analysed TNF-α release into the culture supernatants of cells that were cultured in SFM or medium that was supplemented with 10% human serum or LDS. Based on our previous studies, we selected a concentration of 17 μg/mL SAA (Hatanaka et al. 2004). SAA was pre-incubated with SFM or medium that was supplemented with LDS or human serum for 30 min under mild shaking and subsequently added to the cell cultures. The pre-incubation was performed to allow for possible associations between SAA and serum components. The SAA-induced TNF-α production was measured in the culture supernatants after 16 h of incubation. Cell viability was higher than 90% in all of the conditions assayed, which was verified using the trypan blue exclusion test (data not shown). SAA induced TNF-α release from THP-1 cells in SFM and medium that was supplemented with 10% LDS ($p < 0.05$ and $p < 0.001$ compared to unstimulated cells, respectively). The SAA-induced TNF-α release in SFM was two-fold lower than that in medium that was supplemented with LDS (Fig. 1A). These results indicate the importance of serum components in SAA-induced cytokine production. Conversely, SAA-induced TNF-α production was almost completely abolished in the presence of whole human serum. These findings suggest that serum components play a role in the control of SAA-induced cytokine production. Because SAA circulates in association with HDL, the effects of HDL on SAA-mediated induction of TNF-α were tested. HDL that was isolated from human plasma was pre-incubated with SAA in SFM or medium containing LDS for 30 min and subsequently added to THP-1 cell cultures. In the tested concentration range, HDL did not induce TNF-α production (Fig. 1B, C). In SFM, the lowest concentration of HDL (125 μg of protein/mL) had no effect on SAA-induced TNF-α release, whereas 250 μg of protein/mL and 500 μg of protein/mL of HDL inhibited SAA-induced TNF-α release by approximately 3.5 and 4-fold, respectively (Fig. 1B). We also tested the effects of HDL in culture medium that was

supplemented with LDS. We verified that HDL inhibited the release of TNF-α that was induced by SAA in a dose-dependent manner (Fig. 1C). The concentrations of HDL (125, 250 and 500 µg of protein/mL) that were assayed in the current study are close to the concentrations that are found in the sera of healthy individuals (Naiko 2003).

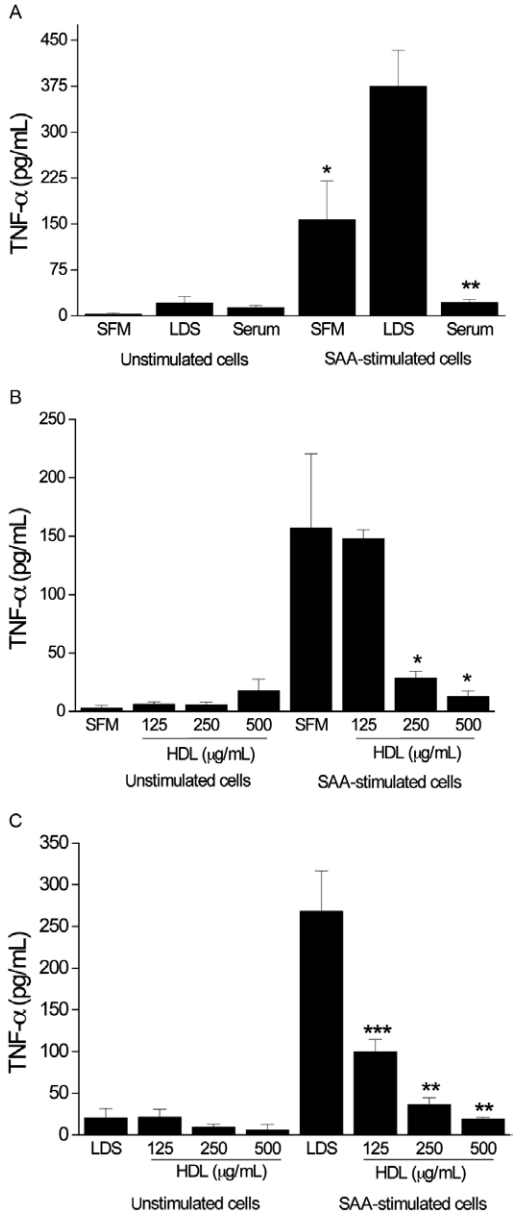

Fig. 1A: serum amyloid A (SAA)-induced tumour necrosis factor-alpha (TNF-α) production in THP-1 cells in the presence of serum-free medium (SFM), medium supplemented with lipoprotein-deficient serum (LDS) or medium supplemented with human serum (*: $p < 0.05$; **: $p < 0.01$ vs. cells incubated with LDS). Effects of high-density lipoprotein (HDL) (µg of protein/mL) at different concentrations on SAA-induced TNF-α release in SFM (B) and LDS-supplemented medium (C) (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs. cells incubated in the absence of HDL). THP-1 cells were stimulated with SAA (17 µg/mL) and incubated for 16 h. The data shown represent the average ± standard error of mean of three independent experiments.

The inhibitory effects of lipoproteins on the action of SAA is limited to HDL - Given that HDL strongly inhibited SAA-induced TNF-α production, we investigated whether LDL or VLDL affected the SAA-induced TNF-α secretion in medium that was supplemented with LDS. Although concentrations of 125, 250 and 500 µg of protein/mL HDL inhibited SAA-induced TNF-α release by approximately 60%, 92% and 97%, respectively (Fig. 2A), LDL and VLDL at different concentrations did not inhibit SAA-induced TNF-α production (Fig. 2B, C).

Effects of human serum and HDL on SAA-induced production of TNF-α and IL-1β in PBMCs - To further verify our results in an alternate cell type we evaluated SAA-induced TNF-α release from PBMCs under the same conditions as those used to assay THP-1 cells. We also assayed the levels of the proinflammatory cytokine IL-1β in PBMCs. As expected, SAA induced the release of TNF-α and IL-1β from PBMCs ($p < 0.001$ and $p < 0.001$ compared to unstimulated cells, respectively). Unlike THP-1 cells, PBMCs were not affected by the absence of serum over the time interval that was assayed. SFM or medium that was supplemented with human serum or LDS did not differentially affect SAA-induced TNF-α production (Fig. 3A), even with the addition of HDL at different concentrations (Fig. 3B). The addition of HDL (250 µg of protein/mL) to the medium containing human serum did not affect TNF-α production induced by SAA (data not shown). However, the addition of HDL to LDS-containing medium promoted a significant decrease in TNF-α release (Fig. 3C). We observed that the SAA-induced IL-1β release from PBMCs was affected by the presence of serum (Fig. 4A). Although no significant differences were detected, the presence of HDL decreased IL-1β production when PBMCs were cultured in SFM (Fig. 4B). Conversely, the inhibitory effect of HDL on IL-1β production was clear and pronounced in medium that was supplemented with LDS (Fig. 4C).

DISCUSSION

In the current paper, we reported the effects of whole serum, lipoprotein-depleted serum and medium that was reconstituted with HDL on SAA-induced release of TNF-α and IL-1β in monocytic cells. THP-1 cells and PBMCs released TNF-α and IL-1β following stimulation with SAA. PBMCs were more responsive to SAA than THP-1 cells. This result is likely associated with the differentiation state and amount of receptors that are expressed by these cells (Zarember & Godowski 2002).

In THP-1 cells, the SAA response was dramatically increased following incubation in lipoprotein-depleted serum and was abolished with incubation in whole human serum. These results suggest that factors that are present in serum may modulate SAA-induced cytokine production. The presence of serum, even delipidated serum, may promote interactions between cells and different types of proteins, as well as other macromolecules and small molecules and mediate different effects on cell integrity and biological responses. The reconstitution of lipoprotein-deficient serum with HDL inhibited SAA-induced TNF-α release in a dose-dependent manner. Other studies have shown that HDL inhibits some SAA-

mediated activities, such as chemotaxis (Badolato et al. 1994), the release of tissue factor (Cai et al. 2007) and IL-8 (Baranova et al. 2010) and TNF-α mRNA expression (Song et al. 2009a, b). Furthermore, we previously demonstrated that acute-phase HDL (HDL-SAA) does not promote the production of TNF-α, IL-1β or IL-8 by human blood neutrophils (Furlaneto & Campa 2000).

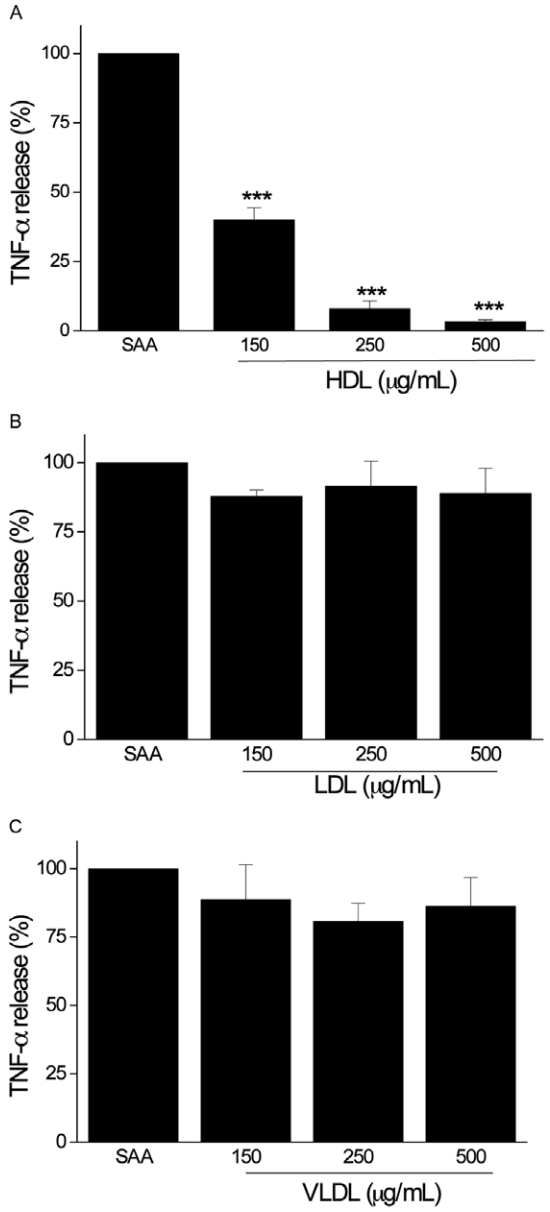

Fig. 2: percentage of inhibition by lipoproteins of serum amyloid A (SAA)-induced tumour necrosis factor-alpha (TNF-α) production by THP-1 cells in medium supplemented with lipoprotein-deficient serum. High-density lipoprotein (HDL) inhibited the production of TNF-α induced by SAA (A). Low-density lipoprotein (LDL) (B) and very LDL (VLDL) (C) (μg of protein/mL) did not affect the release of TNF-α induced by SAA. THP-1 cells were stimulated with SAA (17 μg/mL) and incubated for 16 h. The data shown represent the average ± standard error of mean of three independent experiments. Asterisks mean $p < 0.001$ vs. SAA-treated cells.

In this study, we demonstrated the powerful effects of human serum on the inhibition of SAA activity in THP-1 cells. HDL appears to be one of the main factors that mediate this inhibitory effect. LDL or VLDL did not influence SAA-induced cytokine production by THP-1 cells. Although SAA forms complexes with phospholipids (Bausserman et al. 1983) and other lipoproteins (Marhaug et al. 1982), the absence of an inhibitory effect for LDL and VLDL rules out the possibility that the SAA-induced cytokine production is due to the formation of insoluble aggregates of SAA, which represent a possible structural rearrangement of delipidated SAA, as suggested by Kinkley et al. (2006).

Although the inhibitory effect of HDL on SAA-induced TNF-α production in PBMCs was not as dramatic as that in THP-1 cells, HDL did induce a significant inhibitory effect in PBMCs. In addition to TNF-α inhibition, we observed the inhibition of IL-1β in PBMCs. The reasons for the mitigated inhibitory effect in PBMCs may be related to the high variability in the cell preparations, which depends on the individual donor and the increased responsiveness of PBMCs to SAA compared to that of THP-1 cells.

The inhibitory effect of HDL on SAA-induced proinflammatory cytokine release is an additional anti-inflammatory property ascribed to HDL. The known anti-inflammatory actions of HDL include inhibition of SAA-mediated monocyte tissue factor production (Cai et al. 2007) and the inhibition of C-reactive protein-induced adhesion molecule production, which is another acute-phase protein (Wadham et al. 2004). These aforementioned studies and the results of our study compose the framework of the well-recognised anti-inflammatory effects of HDL. The best-characterised ability of HDL is to scavenge LPS (Munford et al. 1982). When HDL is added to the system, other interactions, including the cooperation between HDL and LPS-binding protein (LBP), are expected. Although the interaction between HDL and LBP has been proven (Massamiri et al. 1997), the complex interactions between HDL and LBP and cell membranes can be estimated by the range of responses that are dependent on the HDL: LBP ratio (Hamann et al. 2005). The anti-inflammatory properties of HDL are not restricted to its scavenging properties. HDL inhibits the expression of adhesion molecules (Cockerill et al. 1995) and induces eNOS activity (Yuhanna et al. 2001). Although these direct effects might be considered in this study, the HDL-mediated inhibition of SAA-induced cytokine production may not be a direct effect of HDL on monocytic cells based on its small effect on the basal production of cytokine (Furlaneto & Campa 2000).

In summary, we previously demonstrated that the ability of SAA to induce the production of cytokines is restricted to its free form because acute-phase HDL (HDL-SAA) does not promote cellular activation (Furlaneto & Campa 2000). At that time, we hypothesised that the HDL particle may serve as a reservoir or transporter of SAA which restricts SAA to specific sites (Furlaneto & Campa 2000, Okino et al. 2006). In this study, we confirmed that HDL suppressed the in vitro proinflammatory activity of SAA and that this effect was not shared with other lipoproteins. It is important to consider that, as stressed by other researchers (Cai et al. 2007, Baranova

et al. 2010), SAA is expressed and synthesised by several extrahepatic sources and may exert biological effects in extravascular sites that are characterised by low concentrations of lipoproteins (Nanjee et al. 2000). These sites include pleural and ascitic exudates (Okino et al. 2006) and inflamed synovial tissue from rheumatoid arthritis patients who exhibit elevated SAA levels (O'Hara et al. 2004). Furthermore, our data suggest that HDL is an additional factor that prevents an unbalanced release of inflammatory cytokines. This role of HDL seems especially important in diseases that are characterised by increased SAA levels and in which TNF-α and IL-1β have important roles, such as atherosclerosis (Libby 2002), obesity (Fantuzzi 2005) and sepsis (Zanotti & Kumar 2002). In all of these diseases, there is evidence suggesting that HDL confers protective effects (Murch et al. 2007).

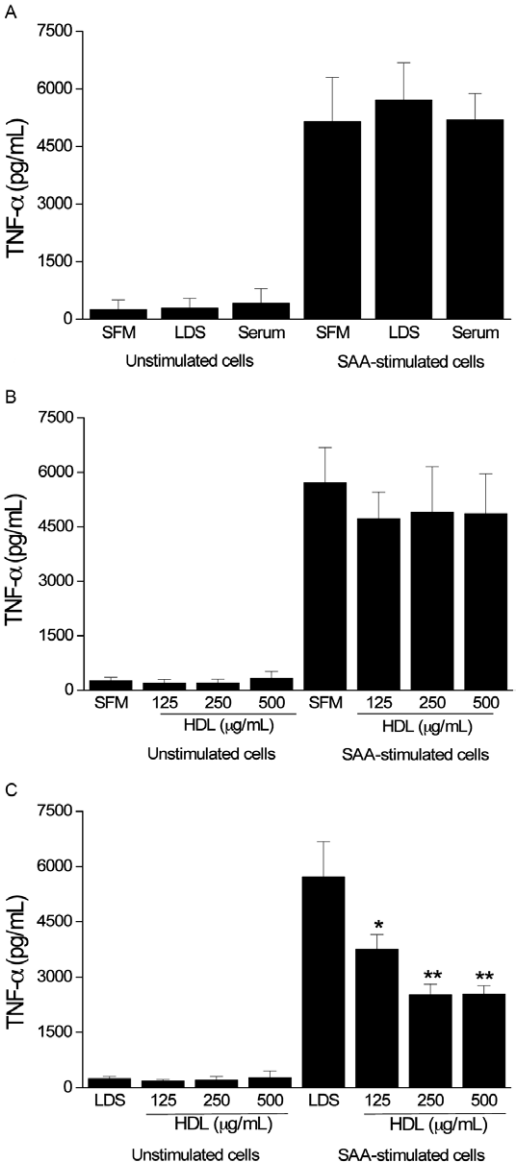

Fig. 3A: serum amyloid A (SAA)-induced tumour necrosis factor-alpha (TNF-α) production in peripheral blood mononuclear cells (PBMCs) in the presence of serum-free medium (SFM), medium supplemented with lipoprotein-deficient serum (LDS) or medium supplemented with human serum. Effects of high-density lipoprotein (HDL) (μg of protein/mL) at different concentrations on SAA-induced TNF-α release in SFM (B) and LDS-supplemented medium (C) (*:$p < 0.05$; **:$p < 0.01$ vs. cells incubated in the absence of HDL). PBMCs were stimulated with SAA (17 μg/mL) and incubated for 16 h. The data shown represent the average ± standard error of mean of six independent experiments.

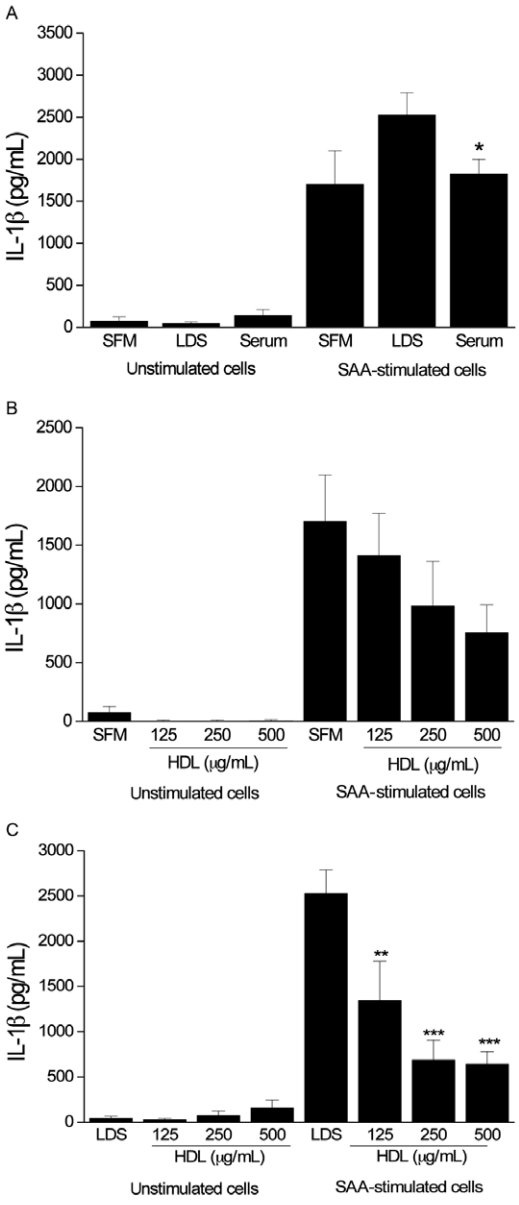

Fig. 4A: serum amyloid A (SAA)-induced interleukin-1 beta (IL-1β) production in peripheral blood mononuclear cells (PBMCs) in the presence of serum-free medium (SFM), medium supplemented with lipoprotein-deficient serum (LDS) or medium supplemented with human serum (*: $p < 0.05$ vs. cells incubated in LDS supplemented medium). Effects of high-density lipoprotein (HDL) (μg of protein/mL) at different concentrations on SAA-induced IL-1β release in SFM (B) and LDS-supplemented medium (C) (*: $p < 0.05$; **: $p < 0.01$ vs. the cells incubated in the absence of HDL). PBMCs were stimulated with SAA (17 μg/mL) and incubated for 16 h. The data shown represent the average ± standard error of mean of seven independent experiments.

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