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High-Density Lipoprotein Attenuates Th1 and Th17 Autoimmune Responses by Modulating Dendritic Cell Maturation and Function

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Aberrant levels and function of the potent anti-inflammatory high-density lipoprotein (HDL) and accelerated atherosclerosis have been reported in patients with autoimmune inflammatory diseases. Whether HDL affects the development of an autoimmune response remains elusive. In this study, we used apolipoprotein A-I–deficient (apoA-I−/−) mice, characterized by diminished circulating HDL levels, to delineate the role of HDL in autoimmunity. apoA-I−/− mice exhibited increased severity of Ag-induced arthritis compared with wild-type mice, and this was associated with elevated Th1 and Th17 cell reactivity in the draining lymph nodes. Furthermore, reconstituted HDL (rHDL) attenuated IFN-γ and IL-17 secretion by Ag-specific T cells upon stimulation of draining lymph nodes in vitro. The suppressive effects of rHDL were mediated through modulation of dendritic cell (DC) function. Specifically, rHDL-treated DCs demonstrated an immature phenotype characterized by downregulated costimulatory molecules, the release of low amounts of proinflammatory cytokines, and failure to promote T cell proliferation in vitro. The mechanism of action involved the inhibition of NF-κB nuclear translocation and the decrease of Myd88 mRNA levels by rHDL.

Finally, modulation of DC function by rHDL was critically dependent on the presence of scavenger receptor class B type I and ATP Binding Cassette Transporter A1, but not the ATP Binding Cassette Transporter G1. These findings reveal a novel role of HDL in the regulation of adaptive inflammatory responses through suppression of DC function that could be exploited therapeutically in autoimmune inflammatory diseases. The Journal of Immunology, 2015, 194: 4676–4687.
ABCA1-dependent manner (19). Although studies have shown that HDL can modulate the activity of various immune cell subsets (12), the mechanism through which HDL regulates autoreactive T cell responses in vivo remains elusive.

Dendritic cells (DCs) are professional APCs that carry Ags in the draining lymph nodes (dLNs) and promote the activation, differentiation, and polarization of naive T cells into effector Th cell subsets (20). Specifically, mature DCs present the Ag in the context of MHC and provide costimulatory signals that are required for efficient activation and priming of T cells. Furthermore, through secretion of proinflammatory cytokines, DCs direct the polarization of T cell toward the different T cell lineages. Ag recognition in the presence of IL-12 favors the generation of Th1 cells, whereas IL-6 and IL-23 drive the generation of Th17 effector cells (21–23). Because Th1 and Th17 cells and proinflammatory cytokines orchestrate the autoimmune responses in RA, strategies aiming at modulation of DC function and subsequent suppression of autoreactive Th1/Th17 responses might provide novel targets in the design of therapeutic protocols for the treatment of this disease.

In this study, we show that rHDL exerts its anti-inflammatory actions through modulation of DC maturation and function, and that rHDL-exposed DCs suppress T cell proliferation in vitro. Of interest, apoA-I L–/– mice that lack conventional HDL develop severe arthritis and elevated Th1 and Th17 cell responses. Finally, our findings support a critical role of SR-BI and ABCA1 transporters in the rHDL-mediated anti-inflammatory function of DCs.

Materials and Methods

Animals

Wild-type (WT) C57BL/6 and apoA-I L–/– (ApoA1tm1Unc) mice on a C57BL/6 background were obtained from the specific pathogen-free experimental protocols were in accordance with institutional guidelines and were approved by the Greek Federal Veterinary Office.

Reagents

Cell cultures were maintained in complete medium consisting of DMEM (Invitrogen), supplemented with 10% FBS (Biochrom) and antibiotics-antimycotic solution (Invitrogen). For flow-cytometric analysis, the following fluorescent-conjugated Abs were used: anti-CD11b (M1/70) and antimycotic solution (Invitrogen). For flow-cytometric analysis, the following fluorescent-conjugated Abs were used: anti-CD11b (M1/70) and anti–MHC class II (MHC-II; AF6-120.1) were purchased from BD Biosciences. Abs against CD3 (145-2C11), CD4 (RM4-5), CD8a (S3-6.7), CD44 (IM7), CD45RB/B220 (RA3-6B2), Ly-6C/EY-6C (Gr-1) (RB6-8C5), CD11c (N418), CD40 (3/23), CD80 (16-10A1), and CD86 (GL-1) were all purchased from Biolegend.

Induction of arthritis and clinical assessment

Mice were immunized s.c. at the base of the tail with 100 µg methylated BSA (mBSA; Sigma-Aldrich) in 50 µl PBS emulsified with equal volume of CFA (Sigma-Aldrich). On day 21 postimmunization, mice were injected intrarticularly (i.a.) with 50 µg mBSA in 20 µl PBS into the left knee joint. The swelling of the joint was evaluated by daily measurement (days 21–30) of the knee diameter (mm) using a digital vernier caliper (Cocraft).

Histological grading

Histological grading of arthritis was evaluated by scoring samples separately for the following parameters: severity of inflammation, extent of epithelial changes, and size of abscesses. A total histological score was assessed by summing the scores (0 = normal, 1 = mild, 2 = moderate, 3 = severe) for each parameter. The range of possible score was 0–9. Histopathology images were captured using a Nikon Eclipse E-400 microscope and Nikon Digital sight, DS-SM, photographic system. Images were autocorrected in Windows Photo Manager.

Serum HDL preparation and cholesterol measurement

Mice were euthanized and blood was collected by cardiac puncture after a 4-h fast. Serum was isolated, and an aliquot was used for HDL preparation. In brief, equal volumes of dextran sulfate (MP Biomedicals) and MgCl2 stock solutions were mixed to produce the precipitation solution consisting of 10 g/dl dextran sulfate and 0.5 M MgCl2. Serum samples were mixed thoroughly with precipitation solution at a volume ratio of 10:1 and incubated for 10 min at room temperature. The mixture was centrifuged for 40 min at 14,000 × g at 4 °C, and the HDL-containing supernatant was collected for determination of cholesterol levels and functionality assays. Total cholesterol and HDL-c were measured using a commercially available assay (Thermo Scientific) according to the manufacturer’s recommendations.

Paraoxonase-1 activity assay

Paraoxonase-1 (PON-1) activity was determined in HDL preparations from mouse serum using paraoxon as substrate. In brief, 5 µl HDL was added in 245 µl buffer (100 mM Tris-HCl, pH 8.0, and 2 mM CaCl2) containing 1.1 mM paraoxon (Sigma-Aldrich), and the absorbance at 405 nm was measured every 40 s for 20 min at room temperature in a microplate spectrophotometer. The rate of p-nitrophenol formed by the hydrolysis of paraoxon was determined by monitoring the increase in absorbance. PON-1 activity was expressed as units per liter (U/l) of HDL, with 1 U defined as the activity that catalyzes the formation of 1 µmol p-nitrophenol per minute.

Preparation of rHDL containing apoA-I

Recombinant apoA-I was produced and purified from the culture medium of adenovirus-infected HTB-13 cells as previously described (26). rHDL particles were prepared by the sodium cholate dialysis method (27) using POPC/cholesterol/apoA-I/sodium cholate in a molar ratio of 100:10:1:100 as previously described (38). The rHDL preparation was extensively dialyzed against PBS, and the formation of apolipoprotein–lipid complexes was verified by two-dimensional gel electrophoresis (29, 30). Concentration of rHDL was based on apoA-I content and was determined by DC Protein Assay (Bio-Rad).

In vitro proliferation of dLN cells

Female mice (8–10 wk old) were immunized s.c. at the base of the tail with 100 µg OVA (Sigma-Aldrich) emulsified in equal volume of CFA. dLNs were excised 9 d later and single-cell suspensions were prepared. dLN cells (dLNCs) were cultured in flat-bottom, 96-well plates (5 × 105 cells/well) in the presence of OVA (15 µg/ml) and/or increasing concentrations of rHDL (1 µM = 28 µg/ml) for 72 h. Cells were then pulsed with 1 µCi [3H]thymidine (TRK120; Amersham Biosciences) for 18 h, and the incorporated radioactivity was measured using a Beckman beta counter. Cyto- kinase levels in culture supernatants were determined by ELISA and cells were analyzed by flow cytometry after 48 h of stimulation.

Generation of mouse bone marrow–derived DCs

Bone marrow–derived DCs (BMDCs) were generated from bone marrow progenitors, as previously described (31). In brief, bone marrow was isolated from femurs and tibias of female mice, treated with RBC lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA), and plated at 2.5 × 106 cells per 100-mm cell culture dish in complete medium supplemented with 20% supernatant from a murine GM-CSF–secreting cell line (X63Ag8; kindly provided by B. Stockinger, National Institute of Medical Research, London, U.K.) (32). The culture medium was half-renewed on days 3, 6, and 8. On day 9, nonadherent cells were collected and cultured in 24-well tissue culture plates (1 × 106 cells/well) in the presence of either 0.5 µg/ml LPS from Escherichia coli D111; B4 (Calbiochem) or 20 ng/ml recombinant TNF-α (Invrospec) and 4 µM HDL. BMDCs were harvested as indicated for RNA, Western blot, and FACS analysis. Culture supernatants were collected at 12 or 18 h for cytokine analysis by ELISA.

Cocultures

BMDCs from WT mice were stimulated with 0.25 µg/ml LPS and pulsed with 20 µg/ml OVA in the presence or absence of 4 µM rHDL for 12 h.
Cells were collected and washed to remove excess LPS, OVA, and rHDL, and were cocultured with dLNcs isolated from OVA-immunized mice (9 d postimmunization) as described earlier. Culture supernatants were harvested 48 h later.

ELISA

Cytokine levels in culture supernatants were measured by ELISA kits following the manufacturer’s recommendations. Mouse IL-17, IFN-γ, IL-12 p70, IL-8, IL-6, and TNF-α were purchased from R&D Systems. Mouse IL-23 and IL-2 were obtained from eBioscience, and IL-10 from Biolegend.

Flow cytometry

Cells were stained by incubation with fluorescent-conjugated Abs for 20 min at 4°C in PBS/5% FBS. Intracellular Foxp3 staining was performed using the Alexa Fluor 488 anti-mouse Foxp3 Ab (Biolegend) and the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), according to the manufacturer’s protocol. Cells were acquired on a FACScalibur (BD Biosciences), and data were analyzed using the FlowJo software (Tree Star).

RNA isolation and quantitative PCR

Total RNA was prepared from mouse BMDCs using TRIzol reagent according to the manufacturer’s protocol (Invitrogen). For each sample, 1 µg isolated RNA was used for cDNA synthesis with SuperScript II Reverse Transcriptase (Invitrogen). Quantitative PCR was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems) using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems). The primer sequences were as follows: mouse Hprt, forward 5′-GGCAAGCTGTCGCTGTTCC-3′ and reverse 5′-GCCAAAATCAACAAGGACTCC-3′; mouse Myd88, forward 5′-CCACCTTGGATGACCCTCT-3′ and reverse 5′-TGGCGGCAACCTTTTCTCT-3′; mouse Trif, forward 5′-TCTACAGCTCTACAAGCCTCCT-3′ and reverse 5′-GTCAAGCTTGTCTTCCATCAA-3′; mouse A1f3, forward 5′-GACCTGAGATGCGCCATCCA-3′ and reverse 5′-CGGCCTCCTTTCCTCATT-3′. Melting curve analysis was performed to ensure primer specificity. The expression of the target genes was normalized to the expression of the housekeeping gene, Hprt, and the normalized threshold cycle (Ct) values were calibrated against the control condition (untreated BMDCs) for each sample. The relative gene expression levels were determined using the relative standard curve method and the comparative Ct method (ΔΔCt) method as described in Applied Biosystems Guide.

Western blot analysis

BMDCs were lysed on ice for 30 min in radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with complete Protease Inhibitor Cocktail (Roche), 5 mM NaF, 1 mM Na3VO4, and centrifuged at 13,000 × g for 30 min at 4°C. The supernatant was collected as total cell lysate, and protein concentration was determined by DC Protein Assay (Bio-Rad). Thirty micrograms total cell protein per sample as total cell lysate, and protein concentration was determined by DC Protein Assay (Bio-Rad). Thirty micrograms total cell protein per sample was denatured by boiling for 10 min, loaded onto a 10.5% SDS polyacrylamide gel, and transferred to nitrocellulose membrane (GE Healthcare Life Sciences). Blots were incubated with primary Abs to anti–p-Stat3 (Cell Signaling) and anti-STAT3 (BD Biosciences). Anti-actin Ab (Merck Millipore) was used as loading control. After incubation with HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories), proteins were visualized on a ChemiDoc XR+ imaging system (BioRad), and band intensities were quantified using the Image Lab Software (BioRad).

Immunofluorescence

BMDCs were seeded onto poly-L-lysine (Sigma-Aldrich)-coated coverslips in 24-well tissue culture plates (1 × 10^5 cells/well). Twenty-four hours later, cells were stimulated with 0.5 µg/ml LPS and treated with 4 µM rHDL for 2 h. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with PBS-Triton X-100 0.5%, and incubated with anti-phospho-Ser70, Ser32 (Santa Cruz Biotechnology). Alexa Fluor 555 Goat Anti-Rabbit IgG (Molecular Probes) was used as secondary Ab. DNA was stained with 1 µg/ml DAPI, and coverslips were mounted on Mowiol 4-88 (Sigma-Aldrich). Images were acquired using a confocal laser scanning microscope (TCS SP8; Leica Microsystems) and an HC PL APO CS2 40×/1.30 oil objective using identical settings within each experiment. Images were obtained using the LAS AF v3.3 software (Leica Microsystems) and processed with Adobe Photoshop 7.0 (Adobe Systems). A total of 100 cells from 4 different fields from each slide was analyzed for fluorescence intensity of nucleus and cytoplasm using the National Institutes of Health ImageJ software.

Statistical analysis

Data are expressed as mean ± SEM unless stated otherwise. Statistical significance was determined using two-tailed Student t test. The χ² test was used for categorical variables. For all results, p < 0.05 was considered statistically significant. Analysis was performed using GraphPad Prism software (version 5.0; GraphPad).

Results

Increased severity of Ag-induced arthritis in apoA-I−/− mice

HDL-c levels are significantly reduced in various autoimmune conditions such as RA (16); however, whether there is a role for HDL in induction and perpetuation of the autoimmune response remains elusive. We investigated the development of Ag-induced arthritis (AIA) in apoA-I−/− mice that are characterized by very low serum HDL-c levels (~25% of normal) (33). AIA is induced upon i.a. injection of mBSA in mBSA-sensitized animals (Fig. 1A) and serves as a model for RA (34, 35). Interestingly, apoA-I−/− mice developed increased severity of AIA compared with WT animals (Fig. 1B). This result correlated well with the histological examination of injected knee joints of apoA-I−/− mice, which developed extensive pannus formation (grade 4), contrary to WT mice, which exhibited moderate-to-severe inflammation (grades 2–3; *p = 0.0393 for pannus versus nonpannus; Fig. 1C, 1D). A striking observation was the extensive inflammatory reaction in the skin of apoA-I−/− mice 3 wk after immunization as opposed to WT mice (Fig. 1E), which was characterized by significant epidermis alterations at the site of the injection including ulceration, dense inflammation in the dermis with abundant neutrophil infiltration, and formation of large abscesses (Fig. 1F, 1G; data not shown). In contrast, WT mice showed moderate inflammatory reaction in the dermis and occasional small neutrophil aggregates (Fig. 1F, 1G). Collectively, these findings indicate that decreased levels of circulating HDL are associated with exacerbation of inflammatory arthritis and severe inflammatory reactions in the skin.

Anti-inflammatory functions of HDL are compromised in arthritic WT mice

We examined whether HDL-c levels were affected by the inflammatory response and whether HDL functionality was altered during AIA development. According to our results, total and HDL-c levels of both mBSA-immunized (day 21) and arthritic (day 30 i.a.) mice showed no significant differences as compared with naive WT mice (Fig. 2A). To assess the functional properties of HDL, we measured HDL-associated PON-1 activity at different time points during disease development. Our results indicated that there is a biphasic response of the immunized mice in terms of HDL activity: an early upregulation of HDL functionality (day 10) that could be accounted for by an early attempt to attenuate the inflammation followed by a gradual decline in HDL functionality as the inflammation resolves (days 21 and 30; Fig. 2B). Importantly, our data showed that upon the i.a. antigenic challenge, HDL functionality significantly decreased to even lower levels (day 21 versus day 30 i.a.; Fig. 2B). Although mice are still capable of responding to the i.a. mBSA challenge by increasing PON-1 activity (day 30 i.a.; Fig. 2B), this potential is markedly compromised compared with day 10 (Fig. 2B). Collectively, our results signify that although the HDL-c levels remained unaffected, the anti-inflammatory/antioxidant properties of circulating HDL were attenuated in an inflammatory environment.

ApoA-I−/− mice exhibit exacerbated OVA-specific Th1 and Th17 immune responses

Development and progression of arthritis depends on the generation of autoreactive Th1 and Th17 cells (36, 37). To monitor the in-
duction of T cell responses in apoA-I−/− mice, we first examined whether T cell lymphopoiesis was affected in these mice. Analysis of CD4+ and CD8+ T cell subsets in thymus and peripheral lymphoid organs revealed no differences between naive apoA-I−/− and WT mice, indicating that loss of apoA-I did not cause alterations in T cell development and homeostasis (Fig. 3A). In addition, the frequency of peripheral CD4+Foxp3+ regulatory T cells (Tregs) was similar in apoA-I−/− and WT mice (Fig. 3B). We next assessed whether decreased HDL levels could affect the induction of Ag-specific Th1 and Th17 responses. Interestingly, dLNs of apoA-I−/− mice showed increased cellularity 9 d after the antigenic challenge compared with WT mice (53.5 ± 6.2.5 versus 42.1 ± 2.0 × 10^6 cells; Fig. 4A). Detailed analysis of the dLNs for the different cell subsets revealed a significant increase of CD11c+ DCs in apoA-I−/− mice, 3.5 and 9 d after the antigenic challenge, compared with WT mice, and to a lesser extent of CD4+ T cells (Fig. 4B). Notably, no significant differences were observed in the frequencies of B220+ B cells and CD11b+ macrophages, whereas CD8+ T cells were slightly decreased in dLNs of apoA-I−/− mice (Fig. 4B). Moreover, the absolute numbers of CD4+Foxp3+ Tregs were significantly increased in immunized apoA-I−/− mice (Fig. 4B). Although both groups of mice exhibited similar frequencies of activated CD3+CD4+CD44+ T cells after in vitro restimulation with OVA (Fig. 4C), dLNCs from apoA-I−/− mice exhibited increased proliferation as indicated by IL-2 levels (181.3 ± 14.1 versus 74.7 ± 7.9; Fig. 4D). This finding was accompanied by markedly increased production of both IL-17 (773.1 ± 105.9 versus 292.5 ± 85.8; Fig. 4E) and IFN-γ (708.9 ± 114.8 versus 172.4 ± 40.0; Fig. 4F) in apoA-I−/− OVA-stimulated dLNC supernatants, whereas IL-10 was undetectable (data not shown). Taken together, these data demonstrate that apoA-I deficiency promotes T cell proliferation and enhances Ag-specific Th1 and Th17 immune responses.

FIGURE 2. HDL functionality is altered during AIA in WT mice. (A) Total (TC) and HDL-c levels in serum of naive mice (day 0), mBSA-immunized mice (day 21), and arthritic mice (day 30 i.a.). (B) Characterization of HDL antioxidant/anti-inflammatory properties by assessment of HDL-associated PON-1 activity (**p = 0.0084, ***p ≤ 0.0007). Results are expressed as mean ± SEM; data are combined from two independent experiments; n = 5–16/group.

FIGURE 1. ApoA-I−/− mice demonstrate an aggravated AIA phenotype. (A) WT and apoA-I−/− mice (n = 13/group) were immunized s.c. with mBSA/CFA, and arthritis was induced upon i.a. injection of mBSA 21 d later. (B) Evaluation of arthritis by measurement of knee joint swelling daily after i.a. injection (n = 8–9/group; day 4: *p = 0.0233, day 5: *p = 0.0455, day 6: *p = 0.0182). Results are expressed as mean ± SEM. (C) Representative H&E-stained sections of inflamed joints from WT (moderate arthritis) and apoA-I−/− mice (pannus), as well as noninjected knee joint (normal), are shown (original magnification ×200). (D) Histological scores for AIA in WT and apoA-I−/− mice. The histopathologies of injected knee joints were scored as described in Materials and Methods; *p = 0.0393 for pannus versus nonpannus. (E) Photos of skin lesions in apoA-I−/− mice 21 d after mBSA immunization. (F) Representative H&E-stained sections of normal and inflamed skin (original magnification ×20 [left panels], ×400 [right panels]). (G) Histological grading of skin inflammation developed in WT and apoA-I−/− mice (*p = 0.0239). In all panels, data are from two independent experiments.
rHDL inhibits Ag-specific Th1 and Th17 immune responses in vitro

Next, we examined whether HDL could directly affect the induction of Th1 and Th17 immune responses. To address this, we performed in vitro restimulation assays of dLNCs from OVA-immunized WT animals in the presence of titrating amounts of rHDL. Treatment with rHDL led to impaired proliferation of OVA-specific T cells as a response to the antigenic stimulus (Fig. 4G). In support, IL-2 secretion was decreased in culture supernatants of rHDL-treated dLNCs (Fig. 4H). Furthermore, decreased proliferation of dLNCs in the presence of rHDL was accompanied by a dose-dependent decrease in secretion of IL-17 (Fig. 4I) and IFN-γ (Fig. 4J). Overall, these results provide direct evidence for a potent role of rHDL in the suppression of Th1 and Th17 immune responses in vitro.

rHDL downregulates costimulatory molecule expression and suppresses proinflammatory cytokine production in stimulated BMDCs

T cell activation, polarization, and proliferation in vivo require Ag presentation by professional APCs. DCs serve as the best candidate for antigen presentation, with zymosan, a TLR2 ligand, and treated them with rHDL. As shown in Fig. 6A, treatment with rHDL led to downregulation of MHC-II, CD40, CD86, and CD80 expression in both dLNs and spleen (Fig. 5). In contrast, no differences were observed in the expression levels of CD80 (Fig. 5). To examine a direct role of HDL on DC maturation, we generated BMDCs from WT mice and treated them with rHDL during LPS stimulation. As shown in Fig. 6A, treatment with rHDL led to downregulation of MHC-II, CD40, CD86, and CD80. To determine whether this effect was specific to the TLR4-mediated response in DCs, we stimulated BMDCs from WT mice with zymosan, a TLR2 ligand, and treated them with rHDL. Although expression of MHC-II was not altered, a significant downregulation of CD40, CD86, and CD80 expression was observed in rHDL-treated zymosan-activated BMDCs compared with zymosan alone (Fig. 6B). These results indicate that the suppressive effect of rHDL on DC maturation is not limited to TLR4-induced immune responses. Furthermore, secretion of proinflammatory cytokines IL-6, IL-12, IL-23, chemokine IL-8, and the anti-inflammatory cytokine IL-10 was reduced in rHDL-treated LPS-stimulated BMDCs, whereas TNF-α production was unaffected by rHDL treatment (Fig. 6C). Notably, rHDL suppressed the secretion of IL-6, IL-8, and TNF-α in immature DCs, indicating that the anti-inflammatory properties of HDL could be exerted independent of LPS. Collectively, these data demonstrate an inhibitory effect of rHDL on activation and maturation of and cytokine secretion by DCs in vitro.

rHDL treatment of mature BMDCs suppresses Ag-specific T cell proliferation

To assess the functional importance of our findings, we performed coculture experiments with rHDL-treated OVA-pulsed BMDCs and OVA-primed dLNCs as shown in Fig. 7A. To this end, WT BMDCs were activated with LPS and pulsed with OVA in the presence or absence of rHDL for 12 h. BMDCs were then cocultured with dLNCs isolated from OVA-immunized WT syngeneic mice. Forty-eight hours later, culture supernatants were collected and IL-2 production was assessed. Interestingly, rHDL-treated OVA-pulsed BMDCs significantly reduced IL-2 production by OVA-primed dLNCs as compared with OVA-pulsed BMDCs (Fig. 7B). These findings provide evidence for the impaired capacity of rHDL-treated DCs to promote T cell proliferation.

rHDL prevents activation of the MyD88-dependent pathway in stimulated BMDCs

LPS binding to TLR4 leads to NF-κB nuclear translocation and induction of inflammatory cytokine gene transcription (39). To gain insights into the molecular events leading to HDL-mediated suppression of DC function, we examined whether rHDL suppressed LPS-induced proinflammatory cytokine secretion by interfering with the NF-κB pathway. To this end, we performed immunofluorescence studies to assess nuclear translocation of the p65 subunit of NF-κB in LPS-stimulated BMDCs. Our findings showed a marked increase in nuclear p65 compared with unstimulated cells, whereas LPS-induced translocation of p65 to the nucleus was impaired after rHDL treatment (Fig. 8A, 8B). In TLR4 signaling, activation of NF-κB is initiated by interactions with either MyD88 or TIR domain–containing adapter-inducing IFN-β (TRIF) adaptor protein (39). To determine whether these two pathways were disrupted by HDL, we assessed mRNA levels in LPS-stimulated BMDCs. We found that rHDL decreased MyD88 mRNA levels in LPS-stimulated BMDCs after 18 h of treatment (Fig. 8C). In contrast, rHDL treatment did not affect Trif expression neither at an early nor at a later time point (Fig. 8D). These data suggest a role for rHDL in modulating inflammatory responses through interference with the MyD88-dependent TLR4 signaling pathway.
FIGURE 4. OVA-specific Th1 and Th17 immune responses are modulated by HDL. (A) LN cellularity in WT and apoA-I/− mice (n = 12–13/group) 9 d after OVA immunization (**p = 0.0018). Results are expressed as mean ± SEM; data are combined from five independent experiments. (B) dLNCs from WT and apoA-I/− mice (n = 4–6/group) were collected 3.5 and 9 d post OVA immunization (p.i.). Gating strategy and frequencies or absolute numbers of CD11c+ DCs, B220+ B cells, CD11b+ macrophages, CD4+ and CD8+ T cells, and Foxp3+ Tregs are shown. Numbers on the gates denote frequencies. Results are expressed as mean ± SEM. *p # 0.0469, **p = 0.0096. (C) Flow-cytometric analysis of OVA-primed dLNCs from WT and apoA-I/− mice (n = 6/group) after in vitro restimulation with 15 μg/ml OVA for 48 h. Gates were set as indicated. Data are representative of three independent experiments. (D–F) Levels of IL-2 (**p < 0.0001), IL-17 (**p = 0.0055), and IFN-γ (**p = 0.0008) in culture supernatants of OVA-primed dLNCs are shown (n = 6–7/group). Results are expressed as mean ± SEM; data are combined from four independent experiments. (G–J) Levels of IL-2 (**p < 0.0001), IL-17 (**p = 0.001, ***p = 0.0006), and IFN-γ (**p < 0.0081) were determined in culture supernatants 48 h later. Results are expressed as mean ± SEM; data are combined from at least three independent experiments.
Recently, activating transcription factor 3 (ATF3), a transcriptional regulator that provides negative feedback on TLR-induced inflammation, was identified as a critical mediator of HDL's anti-inflammatory effects in macrophages (40). Thus, we assessed Atf3 mRNA levels in rHDL-treated BMDCs. Indeed, after 4 h of treatment with rHDL, LPS-stimulated BMDCs demonstrated increased expression of Atf3 compared with nontreated cells, whereas expression was decreased at 18 h (Fig. 8E). This finding suggests that an HDL-inducible, ATF3-dependent mechanism may operate in DCs, acting as a restrainer of the TLR-stimulated response by preventing excessive proinflammatory cytokine production.

Anti-inflammatory effects of rHDL on stimulated BMDCs are mediated through ABCA1 and SR-BI

It is well established that HDL exerts its pleiotropic functions through interactions with SR-BI, ABCA1, and ABCG1 transporters (7, 9). To investigate the contribution of each transporter in rHDL's anti-inflammatory effects on DCs, we assessed cytokine secretion in LPS-stimulated ABCG1<sup>−/−</sup>, ABCA1<sup>−/−</sup>, and SR-BI<sup>−/−</sup> BMDCs in the presence or absence of rHDL. Deficiency in any of these transporters in BMDCs did not affect their ability to efficiently secrete proinflammatory cytokines upon LPS stimulation (data not shown). Interestingly, rHDL suppressed the levels of IL-12, IL-8, or IL-23 in ABCG1<sup>−/−</sup> BMDCs as efficiently as in BMDCs from WT mice and to an even greater extent the secretion of IL-6, indicating that this transporter is not involved in the inhibitory effect of rHDL on DC activation (Fig. 9A). In contrast, rHDL was unable to efficiently impair proinflammatory cytokine secretion in ABCA1<sup>−/−</sup> and SR-BI<sup>−/−</sup> BMDCs, suggesting that rHDL-mediated modulation of DC function is ABCA1 and SR-BI dependent (Fig. 9A). Notably, the decrease in IL-10 levels that was observed upon rHDL treatment of LPS-stimulated WT BMDCs (34.5 ± 1.6%) was retained independent of ABCG1, ABCA1, or SR-BI deficiency (ABCG1<sup>−/−</sup>: 30.5 ± 1.2%; ABCA1<sup>−/−</sup>: 38.0 ± 1.4%; SR-BI<sup>−/−</sup>: 35.9 ± 2.6%; Fig. 9A). This result suggests either that the ABCG1 transporter does not contribute to rHDL's inhibitory actions or that in the case of IL-10 there is a redundancy in the functions of the three transporters.

Further support of these findings was obtained upon assessment of the effects of rHDL on signaling events downstream of the NF-κB pathway in BMDCs from WT mice or mice deficient for any of the above transporters. Upon LPS stimulation, STAT3 signaling, as indicated by increased STAT3 phosphorylation, was efficiently induced in BMDCs from all groups as compared with non-stimulated cells (WT: 6.0 ± 0.8-fold; ABCG1<sup>−/−</sup>: 4.3 ± 0.5-fold; ABCA1<sup>−/−</sup>: 4.2 ± 0.5-fold; SR-BI<sup>−/−</sup>: 3.9 ± 0.5-fold; Fig. 9B, 9C), and treatment with rHDL was able to diminish STAT3 activation in WT (4.5 ± 0.7-fold; *p = 0.0283) and ABCG1<sup>−/−</sup> BMDCs (2.5 ± 0.2-fold; *p = 0.0127). However, rHDL-treated ABCA1<sup>−/−</sup> and SR-BI<sup>−/−</sup> BMDCs demonstrated similar levels of LPS-induced STAT3 phosphorylation (ABCA1<sup>−/−</sup>: 3.9 ± 0.7-fold; SR-BI<sup>−/−</sup>: 4.2 ± 0.7-fold; Fig. 9B, 9C) as compared with cells treated with LPS alone. Overall, our data provide evidence for an important role of ABCA1 and SR-BI, but not ABCG1, in the HDL-mediated anti-inflammatory function on DCs.

Discussion

Although numerous studies have reported the anti-inflammatory properties of HDL, the mechanisms involved in HDL-mediated suppression of autoimmune inflammatory responses are not well understood. Our results demonstrate that HDL suppresses the activation, maturation of, and cytokine secretion by DCs resulting in the establishment of a “semimature phenotype” of these cells. To this end, treatment of BMDCs with rHDL resulted in reduced
expression of MHC-II and costimulatory molecules that are required for efficient Ag presentation and activation of T lymphocytes. This is in line with previous reports revealing a role of HDL and apoA-I on human DC differentiation and function (41, 42). Ag loading to MHC-II is mediated either through the classical or via the autophagy pathway (43, 44). Recent data suggest that HDL inhibits autophagy induced by oxidized low-density lipoproteins in endothelial cells (45). Whether HDL could suppress the induction of autophagy in DCs and delivery of Ags to MHC loading compartments remains to be shown.

The functional importance of the anti-inflammatory effects of HDL on DCs is demonstrated by the decreased ability of rHDL-treated BMDCs to promote T cell proliferation in vitro. DCs with a “semimature phenotype” have the ability to downregulate immune responses and to ameliorate autoimmunity upon adoptive transfer in vivo (46, 47). In particular, it was shown that TNF-α-treated DCs are tolerogenic and suppress experimental autoimmune thyroiditis through induction of CD4+CD25+ Tregs in vivo and in vitro (47). Because Tregs play a prominent role in the re-establishment but also maintenance of self-tolerance, it remains to

FIGURE 6. rHDL prevents activation and maturation of BMDCs. BMDCs from WT mice (n = 2–6) were stimulated with LPS (0.5 µg/ml) or zymosan (20 µg/ml) and treated with 4 µM rHDL for 18 h. (A) Expression levels of MHC-II (*p = 0.026), CD40 (**p = 0.0076), CD86 (*p = 0.0226), and CD80 (**p = 0.0047) were evaluated by flow cytometry. Representative FACS histograms are shown. Gates were set on CD11c+ cells. Dot plots represent the averages of the geometric mean fluorescence intensity (MFI) of at least two independent experiments. (B) Expression levels of MHC-II, CD40, CD86, and CD80 were evaluated by flow cytometry. Representative FACS histograms are shown. Gates were set on CD11c+ cells. (C) Levels of IL-6, IL-23, IL-12 p70, IL-8, IL-10, and TNF-α in culture supernatants were measured by ELISA. Results are expressed as mean ± SEM; data are combined from three independent experiments; *p = 0.0229, **p ≤ 0.0038, ***p ≤ 0.0008. ND, not detectable; ns, not significantly different.
be examined whether rHDL-treated BMDCs possess the ability to induce and/or expand Tregs in vivo. Interestingly, absolute numbers of CD4+Foxp3+ Tregs in the dLNs of immunized apoA-I−/− were increased compared with WT mice. However, the suppressive potential of Foxp3+ Tregs in an inflammatory environment is currently under debate (48, 49).

According to our data, absence of apoA-I in mice resulted in exacerbated inflammation in the knee joints during AIA and caused extensive inflammatory reactions in the skin. These findings are in line with previous studies demonstrating that lack of apoA-I in low-density lipoprotein receptor–deficient mice fed with high-fat diet was associated with skin lesion development due to enlarged skin dLNs that contained expanded populations of cholesterol-enriched lymphocytes including T cells, B cells, and DCs (50). Moreover, when fed an atherogenic diet, these double-knockout mice exhibited increased T cell activation, proliferation, and production of autoantibodies in the plasma. Importantly, this autoimmune phenotype was restored after treatment of mice with either lipid-free apoA-I or adenovirus-mediated gene transfer of apoA-I (50, 51).

The capacity of HDL to bind LPS and neutralize its inflammatory activity has been demonstrated in multiple in vitro and in vivo studies (52, 53). Although this possibility cannot be excluded, the data presented in this study using BMDCs from ABCA1−, ABCG1−, or SR-BI−/− mice did not support such a mechanism of action for rHDL. In our experiments, rHDL did not efficiently suppress LPS-induced secretion of IL-6, IL-12, or IL-23 in mice lacking ABCA1 or SR-BI, indicating that a specific interaction between rHDL and these transporters, rather than sequestration of LPS by rHDL, is responsible for its anti-inflammatory functions on BMDCs.

**FIGURE 7.** rHDL-treated DCs suppress Ag-specific T cell proliferation in vitro. (A) Outline of the coculture experimental setup. LPS-stimulated, OVA-pulsed BMDCs from WT mice (n = 5/sexperiment) were treated with rHDL for 12 h and cocultured with OVA-primed dLNCs. Cells were restimulated with OVA and 48 h later (B) IL-2 secretion in culture supernatants was assessed by ELISA. Results are expressed as mean ± SEM; data are combined from two independent experiments (**p = 0.005).

**FIGURE 8.** rHDL inhibits activation of BMDCs by interfering with the TLR4 pathway. WT BMDCs were stimulated with LPS (0.5 μg/ml) and treated with 4 μM rHDL for the indicated time periods. (A) Confocal immunofluorescence microscopy for p65 (red) translocation into the nucleus (DAPI stained, blue) in BMDCs stimulated with LPS for 2 h. Maximum projections of image stacks are shown; representative of two independent experiments. (B) Quantification of p65 cell distribution by determining nuclear/cytoplasmic (N:C) ratios of signal intensity at single-cell level. Data are combined of two independent experiments; ***p < 0.0001. (C–E) Relative mRNA levels of Myd88 (**p = 0.0004), Trif, and Aft3 (*)p = 0.0177, **p = 0.0016) after treatment of WT BMDCs (n = 6–8) with rHDL for 4 and 18 h. Results are expressed as mean ± SEM; data are combined from at least three independent experiments.
LPS stimulation of DCs activates NF-κB, via either the MyD88- or the TRIF-dependent pathway, which is then recruited to the nucleus to induce inflammatory cytokine gene transcription (39). Our data demonstrate that the anti-inflammatory effects of rHDL on LPS-stimulated BMDCs are mediated through interference with the MyD88/NF-κB pathway. Although the inhibitory actions of apoA-I and HDL on TLR signaling have been previously reported (19, 54), a mechanism by which HDL selectively impedes the MyD88-dependent signaling in DCs has not yet been established. Based on our findings, we speculate that interaction of apoA-I on HDL with cell-surface lipid transporters such as ABCA1 or SR-BI leads to changes in downstream signaling events that, in turn, compromise NF-κB activation and transcriptional upregulation of Myd88 by LPS. This could be mediated via two alternative, but not necessarily mutually exclusive pathways. One mechanism could involve depletion of lipid rafts from plasma membranes as a result of cholesterol efflux initiated by apoA-I/ABCA1 or apoA-I/SR-BI interactions. Lipid rafts are dynamic structures that have been critically implicated in a variety of cellular processes including signal transduction, endocytosis, vesicular transport, and immunoregulation (55). Treatment of APCs with either HDL or apoA-I has been associated with cholesterol depletion and lipid raft disruption leading to suppressed T cell activation and downregulation of proinflammatory cytokine secretion (56, 57). The second mechanism could involve activation of ABCA1- or SR-BI–induced signaling that results in inhibition.
of the TLR-induced response. According to previous studies, interaction of HDL or apoA-I with SR-BI present on endothelial cells activates downstream signaling cascades stimulating a variety of protective cellular responses (58). In addition, apoA-I binding to ABCA1 activates the JAK2/STAT3 pathway in macrophages leading to suppression of LPS-induced inflammatory cytokine production through the action of the mRNA-destabilizing protein, tristetraprolin (59). To date, no signaling events have been reported as a result of interactions between apoA-I or HDL and ABCG1, a transporter known to greatly contribute to the cholesterol efflux process in macrophages. Notably, in our study, the inhibitory properties of HDL on BMDCs were found to be independent of ABCG1. These data argue against the hypothesis that the anti-inflammatory effects of HDL on BMDCs are due to cholesterol depletion leading to lipid raft remodeling and favor a model that implicates transporter-specific downstream signaling events.

In a recent study, ATF3, a transcriptional repressor of TLR-stimulated inflammation, was identified as an HDL-induced target gene that mediated HDL’s anti-inflammatory actions in macrophages (40). It was shown that the protective effects of HDL on TLR responses were fully dependent on ATF3 in vitro and in vivo. We show in this study that treatment of LPS-stimulated BMDCs with rHDL caused an upregulation in ATF3 expression. These data support the notion that an ATF3-dependent mechanism may also be activated by HDL in DCs to constrain the inflammatory response.

In summary, our findings provide evidence for a novel role of HDL in shaping the autoimmune responses both in vivo and in vitro. HDL suppresses adaptive T cell responses by modulating the proinflammatory function of DCs, in an ABCA1- and SR-BI-dependent fashion. The results presented in this article shed light on the proinflammatory function of DCs, in an ABCA1- and SR-BI–dependent fashion. The results presented in this article shed light on the proinflammatory function of DCs, in an ABCA1- and SR-BI–dependent fashion. The results presented in this article shed light on the proinflammatory function of DCs, in an ABCA1- and SR-BI–dependent fashion. The results presented in this article shed light on the proinflammatory function of DCs, in an ABCA1- and SR-BI–dependent fashion. The results presented in this article shed light on the proinflammatory function of DCs, in an ABCA1- and SR-BI–dependent fashion. The results presented in this article shed light on the proinflammatory function of DCs, in an ABCA1- and SR-BI–dependent fashion.
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