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Interleukin 10 Promotes Macrophage Uptake of HDL and LDL by Stimulating Fluid-Phase Endocytosis

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Abstract

Objective: Highly elevated plasma levels of interleukin-10 (IL-10) are causally associated with “Disappearing HDL Syndrome” and low plasma LDL-cholesterol, but the underlying mechanism is poorly understood. Fluid-phase endocytosis, a process highly dependent on actin dynamics, enables cells to internalize relatively high amounts of extracellular fluids and solutes. We sought to investigate whether IL-10 induces lipoprotein uptake by fluid-phase endocytosis in macrophages.

Methods and results: Macrophages (RAW264.7, Kupffer and human) were incubated with vehicle (PBS) or IL-10 (20ng/ml) for 7 days. Uptake of HDL, LDL, and/or fluid-phase endocytosis probes (albumin-Alexa680®, 70 KDa FITC-Dextran and Lucifer Yellow, LY) was evaluated by FACS. Intracellular cofilin and phosphorylated cofilin (p-cofilin) levels were determined by immunoblotting.

Macrophage uptake of lipoproteins and probes was non-saturable and increased after IL-10 incubation ($p < 0.0001$). Furthermore, pre-incubation with fluid-phase endocytosis inhibitors (LY294002, Latrunculin A, and Amiloride) significantly reduced uptake ($p < 0.05$). IL-10 increased the cofilin/p-cofilin ratio ($p = 0.021$), signifying increased cofilin activation and hence filamentous actin. Consistently, phalloidin staining revealed increased filamentous actin in macrophages after IL-10 treatment ($p = 0.0018$). Finally, RNA-seq analysis demonstrated enrichment of gene sets related to actin filament dynamics, membrane ruffle formation and endocytosis in IL-10-treated macrophages ($p < 0.05$). IL-10 did not alter mRNA levels of *Ldlr*, *Vldlr*, *Scarb1*, *Cd36* or *Lrp1*. In primary human monocyte-derived macrophages and murine Kupffer cells, IL-10 incubation also increased uptake of lipoproteins, albumin and LY ($p < 0.01$).

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Conclusions: Interleukin-10 induces the uptake of HDL and LDL by fluid-phase endocytosis by increasing actin-filament rearrangement in macrophages, thus providing a plausible mechanism contributing to “Disappearing HDL Syndrome”.

INTRODUCTION

Interleukin-10 (IL-10) is a homodimeric cytokine, produced by a large variety of cells, including macrophages, B- and T-lymphocytes, and NK cells (1). At moderate levels, IL-10 is widely known for its potent anti-inflammatory properties by limiting host immune response to pathogens, thus minimizing inadvertent damage to the host (2). Surprisingly, it has recently become clear that IL-10 has immune-stimulatory properties as well. This effect may be dose-dependent, providing a potential explanation for the overproduction of inflammatory cytokines in humans treated with high doses of recombinant (r)IL-10 (3).

In “Disappearing HDL Syndrome,” individuals with previously normal HDL-C and triglyceride levels develop severe HDL-C deficiency over time (4). The mechanisms underlying this phenomenon are not completely understood. We recently demonstrated an association between “Disappearing HDL Syndrome” and several types of B-cell disorders, namely B-cell lymphomas and autoimmune lymphoproliferative syndrome (ALPS) (5). Patients with these disorders were noted to have very low levels of plasma HDL-cholesterol, as well as low LDL-cholesterol, and their plasma levels were inversely related to IL-10 levels. In the same study, administration of rIL-10 to psoriatic arthritic patients produced a dramatic decrease in plasma HDL- and LDL-cholesterol, which normalized after cessation of treatment, demonstrating causality (5). More recently, administration of PEGylated rIL-10 also was found to markedly decrease plasma lipoprotein levels in both wild type and *Ldlr*^{-/-} mice (6), clearly showing that the LDL receptor is not necessary for the decrease in plasma lipoproteins. Depletion of phagocytic Kupffer cells in these mice blocked the decrease in plasma lipids induced by IL-10 (6), suggesting a key role of macrophages and Kupffer cells in this process. However, the exact mechanism underlying the decrease in plasma lipids caused by IL-10 remains obscure.

Fluid-phase endocytosis is a receptor-independent endocytic process, in which cells, especially macrophages and dendritic cells, take up extracellular fluid and its solutes (7). Mechanistically, the process involves actin filament rearrangement and the dynamic formation of filamentous actin-enriched protrusions of the cell membrane, known as cell membrane ruffles (8). Actin dynamics, thereby, play an important role in fluid-phase endocytosis. It is not clear, however, whether IL-10 stimulates fluid-phase endocytosis in macrophages, since conflicting results have been reported using different macrophage models. Previously, IL-10 has been shown to stimulate fluid-phase endocytosis in monocyte-derived dendritic cells (9), but in other reports the opposite was found, using human monocyte-derived macrophages (10).

Cofilin, an actin-binding protein that acts as a depolymerization factor, enhances the rate of actin-filament turnover in cells (11). Recent evidence placed cofilin as one of the key regulators of actin dynamics in fluid-phase endocytosis (12). Cofilin activity is regulated by phosphorylation: Ser-3 phosphorylated cofilin (p-cofilin) is the inactive form, while active

non-phosphorylated cofilin can bind to actin filaments, increasing actin-filament turnover (11). Different stimuli, such as growth factors, induce dramatic changes in levels of cofilin and p-cofilin; however, it is not known if IL-10 triggers cofilin activation.

In vitro assessment of fluid-phase endocytosis can be challenging, since there is no specific probe or inhibitor for its evaluation. However, quantitating uptake of naturally fluorescent probes, such as lucifer yellow (LY), and radio- or fluorescent-labelled probes, such as albumin and high molecular-weight dextran, has been used to evaluate fluid-phase endocytosis. Unlike in receptor mediated uptake, probe uptake in fluid-phase endocytosis is linear with respect to probe concentration and time (13) and not inhibited by excess of unlabeled probe. It is also blocked by low temperature (14). In addition, fluid-phase endocytosis is sensitive to inhibition of Na^+/H^+ exchange by amiloride (15), phosphoinositide 3-kinase (PI3K) inhibitors such as LY294002 (16), and actin filament disruptors, such as Latrunculin A and Cytochalasin D (17).

Recently, non-receptor mediated endocytosis has gained importance as a contributing factor in the pathogenesis of atherosclerosis. Macropinocytosis, a non-receptor mediated process similar to fluid-phase pinocytosis, was shown to be involved in the uptake of native LDL by cells in a CD47 and NOX1-dependent pathway (18). In the case of fluid-phase pinocytosis, native LDL was shown to be taken up by this pathway in *Ldlr*^{-/-} mouse macrophages after treatment with M-CSF, leading to foam cell formation (19).

Whether IL-10 induces lipoprotein uptake by fluid-phase endocytosis is not known. In this study, using the RAW264.7 macrophage cell line, primary human monocyte-derived macrophages and primary mouse Kupffer cells, we show that IL-10 does, in fact, stimulate the uptake of HDL and LDL by fluid-phase endocytosis in a cofilin-dependent process that promotes the rearrangement of actin-filaments.

MATERIALS AND METHODS

-Reagents

Recombinant murine IL-10 and recombinant human IL-10 were purchased from Peprotech (Peprotech, Inc., Rocky Hill, NJ). Amiloride, latrunculin A, FITC-dextran (70 KDa), LY and LY294002 were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Alexa Fluor™ 568, Alexa Fluor™ 350 Phalloidin and Bovine Serum Albumin (BSA) - Alexa Fluor™ 680 were purchased from Molecular Probes (Molecular Probes, Eugene, OR). Antibodies were purchased from Genetex (Genetex, Irvine, CA) (anti-mouse cofilin, p-cofilin and beta-actin) or Abcam (Cambridge, MA) (anti-human/mouse CD36, SR-BI, and LRP1). Primer kits for RT-PCR were purchased from Thermo Fisher (Thermo Fisher, Waltham, MA).

-Cell culture

Murine RAW264.7 macrophages (ATCC, Manassas, VA), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 IU/ml of penicillin G, 100 µg/ml streptomycin and 10% (vol/vol) FBS. Cells were incubated in a humidified incubator with 5% CO₂ at 37°C.

-Preparation and fluorescent labeling of lipoproteins

High-density lipoproteins (d : 1.064 – 1.210 g/L) and LDL (d : 1.019 – 1.064 g/L) were isolated from healthy human plasma by preparative sequential KBr density gradient ultracentrifugation ($330,000 \times g$) followed by extensive dialysis at 4° against PBS to remove KBr. Purified HDL or LDL (3 mg protein) were then incubated with Alexa Fluor™ 568 carboxylic acid, succinyl ester (Invitrogen, Carlsbad, CA) for 1 hour at room temperature in the dark. Free unbound dye was separated from lipoprotein-bound dye by extensive dialysis at 4° C against PBS. Labeling efficiency was determined by measuring fluorescence in a sample aliquot in a Perkin Elmer Victor3 1420 Multichannel Counter (Ex/Em: 560/603 nm) and normalized to protein content. Mean fluorescence was 2546 ± 107 units/ μ g of protein for HDL and 3418 ± 300 units/ μ g of protein for LDL.

-Uptake studies

RAW264.7 macrophages were grown in the presence of IL-10 (20 ng/ml) or vehicle for 7 days to 80% confluence prior to uptake studies. For HDL and LDL uptake studies, cells were incubated with the indicated concentrations of fluorescently labeled HDL and LDL in serum-free DMEM containing 0.1% BSA (Sigma-Aldrich) for 4 h at 37°C (for evaluating uptake) or 4°C (as a negative control). Vehicle or IL-10 (20 ng/ml) were maintained in the cell culture media throughout the uptake period in all experiments. Fluid-phase endocytosis probe uptake was performed similarly but with the indicated concentrations of the fluorescent fluid-phase endocytosis probes LY, BSA-Alexa Fluor™ 680 (Molecular Probes) and FITC-Dextran (MW 70,000 Da). Fluorescent BSA uptake was also evaluated in the presence of 5X unlabeled BSA. To test the effects of fluid-phase endocytosis inhibitors, RAW264.7 macrophages were grown in the presence of IL-10 (20 ng/ml) or vehicle for 7 days. After the 7-day IL-10 incubation, the inhibitors (40 μ M LY294002, 30 μ M Latrunculin A, 300 μ M amiloride or vehicle) were added to the cells for 30 minutes. Lipoproteins or fluid-phase endocytosis probes were then added, and uptake was performed as described above, with IL-10 or vehicle and inhibitors present during the uptake period. In all cases, after incubation with fluorescently labeled lipoproteins or probes, cells were washed with PBS and detached for flow cytometry with ice cold 2.5 μ M EDTA in PBS. Uptake, proportional to mean fluorescence intensity, was then quantified by fluorescence activated cell sorter (FACS) flow cytometry in a BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ). The number of counted events was at least 10,000.

-Immunoblot analysis

Protein lysates were prepared from control and IL-10 treated cells by incubation with RIPA buffer in the presence of Halt™ protease and Halt™ phosphatase inhibitors (Thermo Fisher). Western blots were performed as previously described (20) using rabbit anti-mouse cofilin (1:1000), Ser-3 p-cofilin1 (1:1000) and beta-actin (1:15,000) antibodies from GeneTex (Irvine, CA). The secondary antibody was donkey anti-rabbit antibody conjugated to HRP (Abcam, Cambridge, UK). Band intensities for cofilin and p-cofilin were quantified using Fluorchem software (Alpha Innotech Corp, San Leandro, CA) and were normalized the corresponding β -Actin signal. Data is presented as fold-change of IL-10-treated cells in comparison to control (vehicle).

-RNA isolation, expression analysis and RNA-sequencing

RNA from triplicates of control and IL-10 treated cells was extracted using a Qiagen RNeasy® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Residual DNA was removed by on-column DNA digestion, using Qiagen's RNase-Free DNase Set kit (Qiagen). Total RNA concentration was measured using a Nanodrop (ThermoFisher).

To quantitate expression of selected genes by RT-qPCR, cDNA was first synthesized from 100 ng of total RNA, using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara BIO Inc, Kusatsu, Japan). RT-PCR was carried out using TaqMan Gene Expression Assays (Thermo Fisher). *Actb* (Mm02619580_g1) was used as an internal control. The primer kits used for PCR are as follow: LDL receptor: *Ldlr* (Mm01177349_m1), very-low density lipoprotein receptor: *Vldlr* (Mm00443298_m1), *Cd36* (Mm00432403_m1) and *Scarb1* (Mm00450234_m1).

For RNA sequencing, the sequencing libraries were constructed from 100 – 500 ng of total RNA using the Illumina's TruSeq Stranded Total RNA kit with Ribo-Zero (Illumina) following the manufacturer instruction. The fragment size of RNAseq libraries was verified using the Agilent 2100 Bioanalyzer (Agilent) and the concentrations were determined using Qubit instrument (LifeTech). The libraries were loaded onto the Illumina HiSeq 3000 for 2×75 bp paired end read sequencing. The fastq files were generated using the bcl2fastq software for further analysis.

Fastq files were analyzed for gene set enrichment with Partek Flow (Partek Flow, Inc. St. Louis, MO).

-Phalloidin staining of F-actin

For detecting filamentous actin (F-actin), RAW264.7 cells were incubated with 20 ng/ml IL-10 or vehicle for 7 days and then fixed using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol. Cells were then stained for 30 minutes with Alexa Fluor® 350 Phalloidin (Molecular Probes) and extensively washed with PBS. Fluorescence intensity was quantified by flow cytometry as described above. Data is presented as fold-change in phalloidin intensity in comparison to control (vehicle). The number of counted events was 10,000 in each run.

-Fluid-phase endocytosis measurement in primary cells

Human monocyte-derived macrophages—Mononuclear cells were obtained from human donors by monocytopheresis, purified with counterflow centrifugal elutriation and cultured as previously described (21). Monocytopheresis was performed under a human subjects research protocol with informed consent approved by a National Institutes of Health Institutional Review Board. Mononuclear cells were cryopreserved until the moment of use. Before use, cells were thawed and added to 25 ml complete medium (RPMI 1640 medium with 2 mmol/L L-glutamine and 10% FBS) and divided into three groups. The first group of cells was incubated with 50 ng/mL human M-CSF for 9 days. The second group was

incubated with 50 ng/mL human M-CSF plus 20 ng/mL human IL-10 for 9 days, and the third group was incubated with 50 ng/mL human M-CSF alone for 7 days with addition of 20 ng/mL IL-10 on day 7. Medium was changed every 2 days until monocytes differentiated and proliferated sufficiently to become confluent. On day 8, cells were trypsinized and plated at a density of 50,000 cells/well in 24-well plates. On the following day (day 9), uptake experiments were performed. Human macrophages were incubated for 4 h with fluorescent BSA (250 µg/mL), LY (100 µg/mL) and fluorescent HDL (600 µg/mL) and LDL (500 µg/mL). Lipoprotein uptake was also evaluated in the presence of 5X and 10X unlabeled lipoproteins. M-CSF (50 ng/mL) and IL-10 (20 ng/mL) were maintained in the cell culture media throughout the uptake experiments. Uptake, proportional to mean fluorescence intensity, was then quantified by fluorescence activated cell sorter (FACS) flow cytometry in a BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ). The number of counted events was at least 10,000.

Murine Kupffer cells—Kupffer cells were obtained from mouse liver according to Li et al. with slight modifications (22). Briefly, two wild-type C57BL/6J mice were anesthetized and livers were perfused with warm PBS. Livers were rapidly removed and minced into small pieces on ice. Tissue was digested with 0.1% Type IV Collagenase solution in RPMI 1640 for 30 min at 37°C. Kupffer cells were separated from hepatocytes and other cells by sequential centrifugation followed by selective adherence to plastic for 2 hours in DMEM media supplemented with 100 IU/ml of penicillin G, 100 µg/ml streptomycin and 10% (vol/vol) FBS. After culturing Kupffer cells in supplemented DMEM with vehicle (PBS) or IL-10 (20 ng/ml) for 7 days, cells were incubated for 4 h with the fluid phase endocytosis probes LY and BSA-Alexa Fluor™ 680 as described above for RAW264.7 cells. Kupffer cells were washed with PBS, released from the culture surface with 0.25% trypsin – EDTA and resuspended in FACS buffer. Fluorescence intensity was measured by FACS as described above. The number of counted events was 10,000 in each run.

-Statistical analysis

Data is presented as mean ± SD. Differences between groups were tested by Student's t-Test and one-way ANOVA, followed by Dunnett's test, depending on each experiment design or treatment groups. P-values less than 0.05 were considered statistically significant.

RESULTS

Interleukin 10-mediated uptake of HDL and LDL is non-saturable

Figures 1A and B show that relatively low levels of fluorescently labeled native HDL and LDL (50 and 40 µg/ml, respectively) are taken up by RAW264.7 macrophages, and that IL-10 stimulates this process approximately 2-fold (Figure 1 A and B). At these low lipoprotein concentrations, however, receptor-mediated endocytosis could be responsible for the observed uptake. To determine if fluid-phase endocytosis could be a possible mechanism for IL-10 mediated lipoprotein uptake, we repeated this study at very high doses of HDL and LDL to saturate any potential receptors for these ligands. As shown in Figures 1C and 1D, when RAW264.7 macrophages were incubated with either 600 µg/ml of fluorescent tagged HDL and 500 µg/ml of fluorescent tagged LDL, IL-10 treatment of cells was still found to

increase uptake of both lipoproteins ($p<0.0001$) (Figure 1 C and D). To compare lipoprotein uptake at low and high lipoprotein concentrations, uptake in relative fluorescence units can be observed in Supplementary Figure 1.

Dose-response plots for HDL and LDL uptake show complex biphasic curves consistent with uptake by both receptor-mediated processes and by fluid-phase endocytosis (Figure 1 E and F). At lower concentrations the uptake curve started to show evidence for partial saturation, likely due to receptor-mediated uptake, but the uptake curves never completed flattened. Instead, at higher concentrations a near linear increase in cellular uptake was observed for both HDL and LDL, which is consistent with a non-saturable process, such as by fluid-phase endocytosis (Figure 1 E and F). Linear fit for HDL uptake curve revealed a trend towards a greater slope for uptake in the presence of IL-10 (1.01, 95% IC: 0.60 to 1.42 vs. 0.66, 95% IC: 0.49 to 0.85; $p=0.08$). For LDL, linear fit revealed a significantly greater slope for uptake in the presence of IL-10 (3.55, 95% IC: 2.87 to 4.23 vs. 1.92, 95% IC: 1.26 to 2.57; $p=0.0012$). Importantly, uptake of both HDL and LDL was higher with IL-10 than vehicle, at almost all lipoprotein concentrations ($p<0.05$).

Plasma lipoprotein concentration is the result of liver lipoprotein production and lipoprotein clearance. To determine whether IL-10 decreases lipoprotein secretion from liver cells, liver carcinoma cells (HepG2) were incubated with IL-10 for 7 days and apoA-I and apoB secretion over 24 h was measured. IL-10 did not alter apoA-I or apoB secretion ($p>0.20$) (Supplementary Figure 2).

Interleukin 10 increases uptake of fluid-phase endocytosis probes

To further evaluate whether the increased lipoprotein uptake in macrophages induced by IL-10 is consistent with fluid-phase endocytosis, we tested uptake of three known fluid-phase endocytosis probes, namely fluorescent BSA (250 $\mu\text{g/ml}$), LY (100 $\mu\text{g/ml}$) and FITC-Dextran (MW: 70 KDa) (1 mg/ml) (Figure 2 A–C). IL-10 stimulated the uptake of the three fluid-phase endocytosis probes ($p<0.0001$). Notably, uptake of probes, like we observed for HDL and LDL, was about 2-fold higher in cells treated with IL-10 compared to vehicle.

Competition experiments showed that the IL-10-induced albumin uptake, in RAW264.7 macrophages, was not affected by an excess of unlabeled BSA (Supplementary Figure 3), suggesting that uptake would be mediated by a receptor-independent mechanism, such as fluid-phase endocytosis.

The dose-response curve for uptake of these probes was also evaluated in the absence and presence of IL-10. Albumin uptake was linear with concentration in the presence of IL-10 between 100 and 500 $\mu\text{g/ml}$ of BSA (Figure 2 D) and was higher than vehicle at every point ($p<0.01$). Even though albumin uptake in the absence of IL-10 shows a saturable uptake, as an approximation we applied linear fit to both dose-response curves. Linear fits of albumin uptake curves show the increase in uptake in response to increasing albumin concentrations is greater in the presence of IL-10 (curve slopes: 733.2, 95% CI: 461.3 to 1005.0 vs. 248.1, 95% CI: 58.5 to 437.7; $p=0.0035$), indicating that like what we observed for HDL and LDL, a higher albumin uptake is seen in the presence of IL-10, consistent with fluid-phase

endocytosis. In addition, non-linear fit for macrophage albumin uptake in absence of IL-10 can be appreciated in Supplemental Figure 4, showing a saturable uptake curve.

In contrast, for LY, which is thought to be completely water soluble and independent of any receptor, the uptake is almost completely linear with concentration in both the presence and absence of IL-10 and is higher in cells treated with IL-10 compared to vehicle at all points ($p<0.05$) (Figure 2 E). The response to LY dose was consistently higher in the presence of IL-10 (curve slopes: 23.7, 95%CI: 18.1 to 29.2 vs. 15.5, 95% CI 14.7 to 16.4; $p=0.0037$). Similarly, uptake of FITC-dextran (MW: 70 KDa) was linear with concentration and higher in the presence of IL-10 compared to vehicle ($p<0.03$) (Figure 2 F). Moreover, the 70 KDa-dextran uptake dose-response curve showed a greater slope in the presence of IL-10 compared to vehicle (26.13, 95% IC: 23.56 to 28.69 vs. 13.81, 95% IC 13.15 to 14.47; $p<0.0001$). Taken together, these results strongly suggest that IL-10 induces fluid-phase endocytosis in macrophages.

Low temperature blocks IL-10 induced uptake

Fluid-phase endocytosis is highly dependent on temperature, robustly occurring at 37° C but it is negligible at 4° C, whereas some ligand-receptor binding may still occur at 4° C. (14). We, therefore, compared uptake of HDL, LDL and fluid-phase endocytosis probes at 37° C vs. 4° C (Figure 3). Notably, uptake of lipoproteins and probes was significantly decreased at 4° C ($p<0.0001$), being almost undetectable in most cases.

Inhibitors of fluid-phase endocytosis block IL-10 induced uptake

To confirm whether IL-10 induced fluid-phase endocytosis, we tested uptake of lipoproteins and fluid-phase endocytosis probes after pre-incubation with pharmacological inhibitors of key steps of fluid phase endocytosis: LY294002, a PI3K inhibitor (16), and latrunculin, a disruptor of actin polymerization (17). Pre-incubation with either inhibitor significantly reduced the uptake of HDL, LDL, LY and fluorescent albumin ($p<0.001$) (Figure 4 A–D). In addition, fluid-phase endocytosis is also particularly sensitive to amiloride, a Na^+/H^+ exchange blocker, that acts by preventing actin polymerization and membrane ruffle formation (15). Remarkably, the increased uptake of lipoproteins and probes induced by IL-10 was obliterated by amiloride pre-incubation ($p<0.001$) (Figure 4 A–D). These data suggest that mechanistically, IL-10-induced uptake of lipoproteins and probes depends on actin filament polymerization and activation of PI3K pathways. Moreover, uptake is sensitive to amiloride, reinforcing the concept that IL-10 promotes uptake by fluid-phase endocytosis. Unexpected decreased effectiveness in uptake observed with some inhibitors could be due to use of sub-maximal doses and/or off-target effects. Alternatively, IL-10 may have stimulated receptor-mediated uptake, consistent with data shown in Figure 2.

Notably, the inhibitor concentrations used correspond to the highest inhibitor concentrations that did not produce significant reductions in cell viability at experimental conditions (Supplementary Figure 5); thus, non-specific effects from the drug treatments should be excluded. Inhibitors were tested for uptake of fluid-phase probes under control conditions (Supplementary Figure 6), showing a slight decrease in albumin and LY uptake. This effect

would suggest some constitutive fluid-phase endocytosis in RAW294.7 macrophages, which would be increased by IL-10.

Interleukin 10 induced cofilin activation and increased filamentous actin

Fluid-phase endocytosis is highly dependent of actin filament rearrangement. We, therefore, evaluated the expression levels of cofilin and p-cofilin as a regulator of actin filaments assembly and remodeling. The ratio p-cofilin/total cofilin was significantly reduced in IL-10 treated macrophages in comparison to vehicle ($p=0.021$) (Figure 5 A), suggesting that IL-10 induces cofilin activation. Indeed, phalloidin staining demonstrated higher F-actin levels in IL-10 treated macrophages ($p=0.0018$) (Figure 5 B), consistent with fluid-phase endocytosis.

In the attempt to determine the causative role of cofilin, we conducted siRNA interference experiments. Even though cofilin mRNA decreased after 5 days, protein levels were still unchanged (Supplementary Figure 7), probably due to cofilin's long half-life. As a result, no differences were found in dextran uptake (Supplementary Figure 7).

IL-10 does not alter lipoprotein receptor mRNA and protein levels in RAW264.7 cells.

To determine whether IL-10 altered gene expression of potential lipoprotein receptors involved in increased lipoprotein uptake, RNA was isolated from RAW264.7 macrophages treated with IL-10 and select mRNAs for receptor genes were analyzed by qRT-PCR (Figure 6 A–C). No differences were observed in *Scarb1* mRNA levels in cells treated with IL-10 and control ($p=0.15$) (Figure 6 A). Moreover, SR-BI protein expression was also unchanged between groups (Supplemental Figure 8), suggesting that increased HDL uptake in IL-10 treated macrophages is independent of SR-BI. Also, *Cd36* and *Lrp1* mRNA and protein levels in RAW264.7 macrophages did not show differences between treatments ($p>0.47$) (Figure 6 B–C). More interestingly, mRNA levels of *Ldlr* and *Vldlr* were undetectable in both control and IL-10 treated macrophages. Consistently, LDLR and VLDLR protein levels were almost undetectable (Supplemental Figure 8). These results suggest that lipoprotein uptake in IL-10 treated cells would be independent of these receptors.

IL-10 induces actin filament, endocytic and membrane ruffling pathways

To gain further insight into gene expression changes, whole RNA sequence analysis was done before and after IL-10 treatment of RAW264.7 macrophages as is shown in Supplemental Table 1, Supplemental Data. Heatmap representation of the up- and downregulation of the 12,506 detected genes is shown in Figure 7. Gene set analysis showed that IL-10 induced pathways related to immune modulation, cell migration and chemotaxis and response to stimuli. However, we focused on the gene sets that could potentially be associated with lipoprotein uptake in macrophages. The enriched gene sets of interest are shown in Table 1. Interestingly, actin filament-based process, actin cytoskeleton organization and actin binding were among the most enriched gene sets, suggesting a high actin filament dynamic in macrophages in response to IL-10. Also enriched were gene sets for endocytosis, receptor-mediated endocytosis and regulation of endocytosis processes, demonstrating that endocytosis pathways are also stimulated by IL-10. Finally, ruffle membrane and cell projection gene sets were also induced by IL-10. In Figure 8, the direct and indirect potential interactions between IL-10 (blue circle) and IL-10 receptor (red square) with the genes listed

in Table 1, involved in actin filament cytoskeleton organization, endocytosis, regulation of actin cytoskeleton organization and ruffle membrane, are shown.

Interleukin 10 induces fluid-phase endocytosis in primary macrophages

Primary human monocyte-derived macrophages have been previously used to demonstrate LDL uptake by fluid-phase endocytosis (19). After IL-10 incubation for 7 days, human macrophages showed increased number of intracellular vacuoles (Figure 9 A and B), consistent with macropinosomes (19). Similar to RAW264.7 macrophages, uptake of fluid-phase endocytosis probes was increased in human primary macrophages in response to IL-10 incubation for 8 days ($p=0.00001$) (Figure 9 C and D). Remarkably, the increased HDL and LDL uptake ($p<0.0001$) observed in human monocyte-derived macrophages after IL-10 incubation was not affected by adding an excess (5X or 10X) of unlabeled lipoprotein (Figure 9 E and F). A slightly smaller, but still significant, effect was observed in human macrophages after 2 days of IL-10 incubation (Supplementary Figure 9).

As Kupffer cells represent ~80% of all resident macrophages (23), we evaluated the effect of IL-10 on the uptake of lipoproteins and fluid-phase endocytosis probes in Kupffer cells isolated from liver of C57Bl/6 mice. IL-10 stimulated the uptake of fluid-phase endocytosis probes in primary macrophages (Figure 10), similar to what was observed in RAW264.7 macrophages. These results are consistent with the prior report (6) that showed that the increased clearance of HDL and LDL from plasma after IL-10 treatment is due to hepatic uptake by Kupffer cells.

DISCUSSION

In the present study, we investigated whether IL-10 stimulates uptake of HDL and LDL in macrophages by inducing fluid-phase endocytosis, as a possible mechanism that could at least explain in part “Disappearing HDL Syndrome”. We found that IL-10 enhances the uptake of both HDL and LDL in macrophages, and for the first time we demonstrate that IL-10 induces HDL and LDL uptake in macrophages by receptor-independent fluid-phase endocytosis. Also, our results show that IL-10 modulates the activity of regulators of actin filament dynamics, such as cofilin among others, leading to an increase in F-actin content in cells. This is supported by RNAseq data. Finally, we demonstrate once again the important role of Kupffer cells in IL-10-induced fluid-phase endocytosis. Our results, therefore, suggest a plausible mechanism for the previously observed “Disappearing HDL syndrome” induced by elevated IL-10 levels (5) and suggests also a potential link between IL-10 and atherosclerosis, although recent human studies have not shown a definitive role of HDL in atherosclerosis (24).

Interleukin-10 is a cytokine known for its anti-inflammatory actions; however, its levels may also sometimes be elevated in pathological conditions, such as in lymphoma (5). Previous studies have evaluated the effect of 6 to 24 hours of IL-10 incubation on *in vitro* lipoprotein uptake in macrophages (25, 26), yielding conflicting results regarding uptake of modified LDL. However, it is important to note that a pathological increase of IL-10 might result in chronic exposure rather than acute exposure, causing a different phenotype. Herein, we evaluated the effect on lipoprotein uptake in macrophages after 7 days of incubation with

IL-10 (20 ng/mL), resembling a chronic exposure to IL-10. Interestingly, incubation of primary human macrophages with 100 ng/mL of IL-10 for 7 days induced a different gene activation pattern than 3 days incubation, favoring cell trafficking and recognition of microbial pathogens (27). It has also been shown that incubation for 7 days with 20 ng/mL of IL-10, rather than 2 days, induced albumin uptake in dendritic cells, presumably by fluid-phase endocytosis (9).

Literature regarding the effect of IL-10 on macrophage uptake of lipoproteins is scarce and discrepant. Some reports show that IL-10 increased uptake of oxidized or acetylated LDL in macrophages (26, 28), which was attributed to increased expression of scavenger receptors such as CD36 (28). On the other hand, Rubic T *et al.* found that uptake of oxidized LDL in THP-1 macrophages was lower in the presence of IL-10 than lipoprotein alone, and this was linked to lower *CD36* expression (25). Inconsistencies could be due to the use of different cell lines, IL-10 concentration and incubation times. However, none of these studies have directly addressed whether IL-10 affected uptake of native lipoproteins. Our results show for the first time that IL-10 increases uptake of native HDL (50 µg/ml), as well as native LDL (40 µg/ml), by RAW264.7 macrophages. Under our experimental conditions, IL-10 did not alter either mRNA or protein expression levels of SR-BI or CD36. Moreover, LDLR and VLDL expressions were almost undetectable in our study. To date there is no agreement whether IL-10 induces specific lipoprotein receptors in macrophages. Thus, IL-10 might induce HDL and LDL uptake in macrophages by other mechanisms than only receptor-mediated endocytosis.

Studies evaluating native LDL uptake in fibroblasts have shown that saturation of receptor-mediated LDL uptake occurs at ~50 µg/ml of lipoprotein (29, 30). So far, all studies aimed to evaluate the effect of IL-10 on lipoprotein uptake have used that lipoprotein concentration to evaluate macrophage uptake, with conflicting results. However, cells such as macrophages and dendritic cells also use fluid-phase endocytosis as a non-specific, receptor-independent mechanism for the internalization of extracellular fluid (7). To determine whether IL-10 induces lipoprotein uptake by fluid-phase endocytosis, it would be necessary to over-saturate receptor capacity. In our study, we evaluated HDL and LDL uptake at very high concentrations, about 10 times receptor saturating concentration: 600 µg/ml and 500 µg/ml for HDL and LDL, respectively.

It has already been established that native LDL can be incorporated into macrophages by fluid-phase endocytosis in response to several stimuli. M-CSF stimulation of human primary macrophages and murine bone-marrow derived macrophages led to native LDL uptake by fluid-phase pinocytosis and foam cell formation (19, 31). More recently, Csányi *et al.* showed that thrombospondin-1 induced, in a CD47 dependent manner, membrane ruffle formation and uptake of native LDL by fluid-phase endocytosis in RAW264.7 macrophages (18). In our study, IL-10 stimulated macrophage uptake of HDL and LDL at very high lipoprotein concentrations, in both RAW264.7 cells and human monocyte-derived macrophages. It should be noted that these lipoprotein concentrations are still in the lower range of normal plasma lipoprotein concentrations. Moreover, macrophage lipoprotein uptake was not saturated in the presence of IL-10. Notably, non-saturable uptake of native LDL has been attributed to occur by fluid-phase endocytosis (32). The steeper slope for HDL and LDL

uptake in the presence of IL-10, observed in our study on RAW264.7 macrophages, reveals that IL-10 induces a more dynamic lipoprotein uptake in macrophages. Furthermore, the lack of uptake inhibition by excess of unlabeled lipoproteins, supports the concept of receptor-independent uptake induced by IL-10. Thus, chronically elevated levels of IL-10 might induce native lipoprotein uptake in macrophages by fluid-phase endocytosis.

Although there is no ideal probe for fluid-phase endocytosis, uptake of some soluble molecules has been used as a metric of this endocytic process. First, we evaluated albumin uptake by macrophages, finding that IL-10 induces macrophage albumin uptake in an apparent non-saturable manner. Similar results were reported by Longoni *et al*, after the same incubation time, in primary dendritic cells (9). Another tracer widely used to monitor fluid-phase endocytosis are LY (33) and high molecular weight dextran (70 KDa). We evaluated the LY and dextran uptake after IL-10 treatment, finding similar results as those observed with albumin. Consistently, all probes presented about 2-fold increase in uptake after IL-10 treatment. Similar results were obtained with human monocyte-derived macrophages treated with IL-10. Importantly, herein human macrophages were differentiated using M-CSF, leading to a M2 phenotype which, unlike M1 phenotype, is known to be prone to constitutive and stimulated fluid-phase endocytosis (34). It is also noteworthy that the uptake slope for the three probes was steeper for IL-10-treated cells, suggesting a more dynamic uptake of probes after IL-10 incubation, as observed for HDL and LDL. This reinforces the idea that IL-10 stimulates fluid-phase endocytosis in macrophages, and this could explain the increase in HDL and LDL uptake observed at very high lipoprotein concentration.

Low temperature has shown to inhibit fluid-phase endocytosis by increasing membrane rigidity (14). Our results show that incubation at 4° C effectively blocks the IL-10 induced uptake of HDL and LDL, and fluid-phase endocytosis probes. However, it should be considered that low temperature can also partially reduce receptor-mediated endocytosis (35).

Several pharmacological inhibitors have been used for the *in vitro* evaluation of fluid-phase endocytosis process, targeting key steps in the process. Fluid-phase endocytosis is known to start with the formation of plasma membrane protrusions (ruffles), rich in actin filaments. PI3K activity is essential for the closure of the cap of ruffle protrusions, allowing their fusion and the formation of intracellular vacuoles (16). In our study, a PI3K inhibitor, LY294002, significantly reduced IL-10 induced uptake of HDL and LDL, as well as fluid-phase endocytosis probes. Interestingly, beside the widely known IL-10/IL-10 receptor/STAT3 pathway; IL-10/IL-10 receptor also activates the PI3K/Akt pathway (36). Therefore, we hypothesize that IL-10 might stimulate fluid-phase endocytosis in RAW264.7 macrophages in part through activation of PI3K. On the other hand, unlike receptor-mediated endocytosis, fluid-phase endocytosis is inhibited by amiloride, a blocker of Na⁺/H⁺ exchange (15). Mechanistically, amiloride lowers sub-membranous pH, preventing actin polymerization and ruffle formation (15). Herein, we show that IL-10-induced lipoprotein, as well as albumin and LY, uptake is blocked by amiloride. These results, therefore, further support the idea that IL-10 induced HDL and LDL uptake by fluid-phase endocytosis in macrophages.

Fluid-phase endocytosis is a process that is highly dependent of actin filaments. A ring of highly polymerizing actin forms beneath the cell membrane, forming a circular ruffle that closes at the top to engulf extracellular fluid (37). In our study, pre-incubation of macrophages with latrunculin A, a potent actin filament disruptor (38), blocked uptake of HDL and LDL and of fluid-phase endocytosis probes in IL-10 treated macrophages, demonstrating for the first time the dependence of IL-10-induced uptake of lipoproteins on actin polymerization, consistent with fluid-phase endocytosis.

Among other actin binding proteins, cofilin regulates actin filament dynamics. Active, Ser-3 dephosphorylated, cofilin acts on the severance of actin filaments, favoring the generation of new actin filaments by providing new free actin monomers (39). We found that IL-10 induces cofilin activation in macrophages, by inducing cofilin Ser-3 dephosphorylation. Recent evidence suggests that cofilin activity has a role in actin filament turnover, occurring in membrane ruffles during macropinocytosis (12). Interestingly, in line with this, we found a modest increase in total F-actin levels in IL-10 treated macrophages. To assess the exact role of cofilin in IL-10 induced actin dynamics and fluid-phase endocytosis, further studies with the generation of stable knock-out cells should be conducted.

There is limited knowledge on the effects of IL-10 on the macrophage transcriptome. In a previous report, using mRNA expression arrays, Jung *et al* analyzed the transcriptome of human peripheral monocytes after different incubation times with IL-10 (10 ng/ml), between 1 h to 35 h, finding up-regulation of genes related to phagocytosis (40). Herein, after 7 days of incubation with 20 ng/ml IL-10, we obtained similar results by using next generation RNA sequencing (Supplemental Table 1). Although in our study, fluid-phase endocytosis gene set did not appear among the most enriched ones, gene sets related to actin filament dynamics and membrane ruffle formation were among the most enriched ones. It is important to note that by interaction analysis we observed possible potential direct and indirect interactions between IL-10 and IL-10 receptor with these processes that are the fundamental mechanisms for fluid-phase endocytosis (41). Our data also shows that genes sets related to receptor-mediated endocytosis are also stimulated by IL-10 in macrophages. We, therefore, cannot exclude the possibility that IL-10 might induce lipoprotein uptake in macrophages by not only receptor-independent fluid-phase endocytosis but also possibly by receptor-mediated endocytosis.

Kupffer cells are the resident macrophages in liver, representing 80 to 90% of all resident macrophages (23). These highly phagocytic cells are in close contact with hepatocytes and represent the first barrier for material passing from the gastrointestinal tract (42). Kupffer cells are sensitive to several stimuli, such as gut flora, fatty acids and cytokines (43); however, very few data exist regarding the effect of IL-10 on Kupffer cells. Herein, our results suggest that IL-10 induces fluid-phase endocytosis in murine primary Kupffer cells *in vitro*. Previously, Chan and coworkers demonstrated that injection of rIL-10 into mice dramatically drops plasma lipid levels, and that effect was obliterated by the depletion of phagocytic Kupffer cells by clodronate administration (6), revealing a key role of Kupffer cells and other macrophages in lipoprotein clearance. Taken together, these results suggest that chronic exposure of macrophages to high systemic levels of IL-10 may induce fluid-

phase endocytosis in Kupffer Cells and other macrophages, promoting the uptake of HDL and LDL, which would contribute to the drop in their plasma levels.

In our previous study, in adults with B-cell lymphoma, IL-10 and triglyceride decreased concomitantly after treatment (5). Importantly, our current data suggest that lipoprotein secretion may not be affected, as IL-10 did not alter apoA-I or apoB secretion from cultured hepatoma cells. Interestingly, in patients with familial low HDL disorders, such as Tangier Disease and Familial LCAT Deficiency, plasma triglycerides are elevated, just as in our lymphoma patients with “Disappearing HDL Syndrome” (5). The mechanism for elevated triglycerides in Tangier disease has been shown to be accumulation of apoA-II in VLDL, causing inhibition of lipolysis by lipoprotein lipase (44). ApoA-II is secreted independently of apoA-I containing HDL particles and jumps on these particles in the plasma after they are secreted (45). In the absence of HDL, apoA-II does not remain free in plasma but instead associates with the available lipoproteins which in apoAI deficient states is VLDL. Nevertheless, we cannot exclude a direct effect of IL-10 on lipoprotein lipase activity, causing accumulation of triglyceride-rich lipoproteins. Additionally, it is still possible that cytokines released by Kupffer cells in response to IL-10-induced lipid uptake might alter hepatocyte lipid metabolism. Further studies are necessary to determine the interaction between Kupffer cells, hepatocytes and lipoprotein metabolism in this context.

In general, fluid-phase endocytosis is considered a non-specific endocytic process. In the context of an increase in fluid-phase uptake by macrophages, stimulated by IL-10, all plasma proteins would be internalized equally. However, certain plasma components, such as immunoglobulins and albumin, have recycling mechanisms that salvage them from degradation. Interestingly, the neonatal Fc receptor has been shown to recognize and bind pinocytosed immunoglobulin and albumin in the acidic endosomal compartment, mediating their recycling back to circulation (46). Other endocytosed proteins lacking recycling pathways, such as lipoproteins, are directed to lysosomal degradation.

We did not address in our study the role of IL-10 in atherogenesis. Other cell-based studies, however, have revealed a potential anti-atherogenic role of IL-10 (26, 47). Several clinical studies have also found that, independent of other risk factors, increased levels of IL-10 is associated with increased risk of primary and secondary cardiovascular events (48, 49). The main clinical impact thus far of “Disappearing HDL Syndrome” is as a potential early warning symptom of lymphoma.

In conclusion, our results demonstrate that IL-10 increases the uptake by macrophages of native lipoproteins by receptor-independent fluid-phase endocytosis. IL-10 induces activation of cofilin, favoring actin filament turnover, membrane ruffle formation and the uptake of lipoproteins in the extracellular fluid by fluid-phase endocytosis. Our findings, therefore, provide a plausible mechanism that could partly explain the inverse association between plasma levels of HDL and LDL with IL-10 observed in “Disappearing HDL Syndrome”.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- IL-10 induces the uptake of HDL and LDL in macrophages by fluid-phase endocytosis.
- IL-10 activates cofilin activity and increases actin filament turnover in macrophages.
- IL-10-induced uptake of HDL and LDL by fluid-phase endocytosis may explain, in part, “Disappearing HDL Syndrome”

Figure 1: Macrophage uptake of lipoproteins was non-saturable and increased by IL-10.

RAW264.7 macrophages were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days, and then incubated with high-density lipoprotein (HDL) or low-density lipoprotein (LDL) for 4 hours. Lipoprotein uptake was analyzed by FACS. Panels (A) and (B) show uptake at low lipoprotein concentration: 50 $\mu\text{g/mL}$ of HDL and 40 $\mu\text{g/mL}$ of LDL. Panels (C) and (D) show uptake at high lipoprotein concentration: 600 $\mu\text{g/mL}$ of HDL and 500 $\mu\text{g/mL}$ of LDL. Bars represent fold change uptake of the control (vehicle). Panel (E) shows HDL uptake from 0 to 600 $\mu\text{g/mL}$ of HDL. Panel (F) shows LDL uptake from 0 to 500 $\mu\text{g/mL}$ of LDL. Each point represents mean $\pm$ SD of three independent experiments, performed in triplicates. * $p < 0.05$; Student's t-test. Insets in panels E and F show linear fits to the corresponding uptake data.

Figure 2: Macrophage uptake of fluid-phase endocytosis probes was non-saturable and increased by IL-10.

RAW264.7 macrophages were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days, and then incubated with increasing concentrations of Alexa Fluor-labeled albumin, lucifer yellow and FITC-labeled dextran (70 KDa) for 4 hours. Uptake of albumin, lucifer yellow and dextran (70KDa) was analyzed by FACS. Panels (A), (B) and (C) show uptake of albumin (250 µg/mL), lucifer yellow (100 µg/mL) and dextran (1 mg/mL), respectively. Bars represent fold change uptake of the control (vehicle). Data is mean $\pm$ SD. * $p < 0.001$; Student's t-test. Panels (C), (D) and (E) show albumin, lucifer yellow and dextran uptake at different concentrations: albumin (0 to 500 µg/mL), lucifer yellow (0 to 200 µg/mL) and dextran (0 to 1 mg/mL). Each point represents mean $\pm$ SD of three independent experiments, performed in triplicates. * $p < 0.001$, ** $p < 0.05$; Student's t-test. Insets in panels D-F show linear fits to the corresponding uptake data.

Figure 3: Incubation at 4°C significantly decreased uptake of native HDL and LDL and fluid-phase endocytosis probes (albumin and lucifer yellow).

RAW264.7 macrophages were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days, and then incubated with: (A) HDL (600 µg/ml), (B) LDL (500 µg/ml), (C) albumin (250 µg/ml) and (D) lucifer yellow (100 µg/ml) for 4 hours, at 37°C and 4°C. Uptake was analyzed by FACS. Bars represent fold change uptake of the control (vehicle), incubated at 37°C. ‡ p<0.0001 vs vehicle (37°C); † p<0.0001 vs. IL-10 (37°C); * p<0.0001 vs. vehicle (37°C). One-way ANOVA.

Figure 4: Fluid-phase endocytosis inhibitors reduced the uptake of lipoproteins and fluid-phase endocytosis probes.

RAW264.7 macrophages were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days, and pre-treated with LY294002 (40 µM, 30 min), latrunculin A (30 µM, 30 min) or amiloride (300 µM, 30 min). Macrophages were then incubated with HDL (600 µg/ml) (Panel A), LDL (500 µg/ml) (Panel B), albumin (250 µg/ml) (Panel C) and lucifer yellow (100 µg/ml) (Panel D) for 4 hours, at the presence of inhibitors. Uptake of lipoproteins and probes was analyzed by FACS. Bars represent ratio of uptake in IL-10-treated cells/uptake in the control (vehicle)-treated cells. * $p < 0.0001$ vs vehicle; † $p < 0.05$ vs. IL-10; One-way ANOVA.

Figure 5: IL-10 induced cofilin activation and an increase in filamentous actin (F-actin) in RAW264.7 macrophages.

RAW264.7 macrophages were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days. RAW264.7 macrophages were lysed, and protein extract was processed by Western Blot for Ser-3 phosphorylated cofilin (p-cofilin) and total cofilin expression (Panel A). Bars show fold change in p-cofilin to total cofilin ratio (Panel B). RAW264.7 macrophages were fixed and stained for F-actin with Alexa-Fluor350 Phalloidin. Quantification was assessed by FACS. Bars represent fold change uptake of the control (vehicle) (Panel C). Data is mean $\pm$ SD. * $p < 0.001$; Student's t-test.

Figure 6: Expression of lipoprotein related receptors and transporters in RAW264.7 macrophages after IL-10 incubation.

RAW264.7 macrophages were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days. mRNA expression of *Scarb1* (A), *Cd36* (B), and *Lrp1* (C) were determined by RTPCR. Bars represent fold change uptake of the control (vehicle). Data is mean $\pm$ SD. * $p < 0.001$; Student's t-test.

Figure 7: Heatmap of normalized expression matrix of the six RNA-seq libraries.

RAW264.7 macrophages were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days (3 independent samples for control and 3 independent samples for IL-10 treated RAW264.7 cells). Total RNA was extracted from cells, libraries were prepared and RNA sequencing was run in a Illumina HiSeq 3000. Data, corresponding to 12,506 genes, was normalized as Fragments Per Kilobase of transcript per Million (FPKM) mapped reads. Green color indicates gene downregulation whereas red color indicates gene upregulation.

Figure 8: Potential interactome of IL-10 and IL-10 receptor with genes listed in Table 1. Interactome was constructed with IL-10, IL-10 receptor and those genes involved in the gene sets listed in Table 1, derived from RNA-seq analysis, using STRING database (<http://string-db.org/>) with moderate confidence (0.40). Gene sets are distinguished as follows: Actin cytoskeleton organization (cyan spheres), phagocytosis (blue spheres), endocytosis (green spheres), regulation of actin cytoskeleton organization (yellow spheres), ruffle membrane (dark green spheres) and ruffle organization (light blue spheres). Genes that share different gene sets appear in all the different colors. IL-10 is highlighted in a blue circle and IL-10 receptor in a red square.

Figure 9: IL-10 induced vacuolization and increased uptake of HDL, LDL and fluid-phase endocytosis probes in human monocyte-derived macrophages.

Human monocyte-derived macrophages were divided into 2 groups. The first group of cells (“M-CSF”) was incubated with 50 ng/mL human M-CSF for 9 days. The second group (“M-CSF + IL-10”) was incubated with 50 ng/mL human M-CSF plus 20 ng/mL human IL-10 for 9 days. On day 7, cells were imaged by phase-contrast microscopy (Panels A and B). IL-10 induced a greater number of vacuoles (red arrows) in macrophages, compatible with macropinosomes (19). On day 9, human macrophages were incubated for 4 h with fluorescent BSA (250 µg/mL), LY (100 µg/mL) and fluorescent HDL (600 µg/mL) and LDL (500 µg/mL). Lipoprotein uptake was also assessed in the presence of a 5X or 10X excess of unlabeled lipoprotein. Uptake was quantified by FACS. * $p < 0.001$ vs. vehicle; one-way ANOVA.

Figure 10: Interleukin 10 increased the uptake of fluid-phase endocytosis probes in primary Kupffer cells.

Primary Kupffer cells were treated with vehicle (PBS) or 20 ng/ml of IL-10 for 7 days, and then incubated with HDL (600 μ g/ml) (Panel A), LDL (500 μ g/ml) (panel B), albumin (250 μ g/ml) (Panel C) and lucifer yellow (100 μ g/ml) (Panel D) for 4 hours. Uptake of albumin and lucifer yellow was analyzed by FACS. Bars represent fold change uptake of the control (vehicle). Data is mean $\pm$ SD. * p <0.02; Student's t-test.

Table 1:

Focused RNA sequencing gene sets, showing those gene sets that could potentially be associated with lipoprotein uptake in macrophages.

Gene set	Description	Enrichment score	P-value	Genes
GO:0032970	regulation of actin filament-based process	10.0757	4.21E-05	<i>Gm14137, Kiss1r, Pfn2, Tnnt2, Plek, Sorbs3, Rhod, Sdc4, Vangl2, Arhgef19, Cdc42ep2, Fgr, Id1, Siglec15, Avil, Csf3, Edn1, Eps8, Fscn1, Rasa1, Mylk2, Il1a, Baiap2, Sema3e, Plekhh2, Kank3</i>
GO:0032956	regulation of actin cytoskeleton organization	10.0704	4.23E-05	<i>Gm14137, Kiss1r, Pfn2, Plek, Sorbs3, Rhod, Sdc4, Vangl2, Arhgef19, Cdc42ep2, Fgr, Id1, Siglec15, Avil, Csf3, Edn1, Eps8, Fscn1, Rasa1, Il1a, Baiap2, Sema3e, Plekhh2, Kank3</i>
GO:0003779	actin binding	7.75045	0.00043055	<i>Lsp1, Myh3, Synpo2, Slc6a4, Pfn2, Tnni2, Tnnt2, Ophn1, Trpv4, Tpm2, Ang, Arc, Coro6, Spata32, Aif1, Ccr5, Xirp1, Avil, Eps8, Fscn1, Sntg1, Myo1h, Rcsd1, Plekhh2, Marcks, Eps8l2, Mefv</i>
GO:0045807	positive regulation of endocytosis	7.67065	0.00046632	<i>Fcgr1, Fcgr3, C3, Axl, Cd14, Ccl2, Sirpa, Dab2, Gas6, Pros1, Ptpn5, Sirpb1a, Mertk</i>
GO:0032587	ruffle membrane	7.37618	0.00062599	<i>Plek, Trpv4, Fgr, Aif1, Eps8, Sntg1, Pacsin1, Eps8l2</i>
GO:0008289	lipid binding	7.35943	0.00063656	<i>Adap2, C3, Ffar4, Ltc4s, Wipi1, Pfn2, Arhgap26, Zfyve28, Plek, Psd4, S100a8, Ophn1, Syt12, Rorc, Trem3, Osbp2, Phlda3, Syt8, C8g, Cd6, Axl, Esyt3, C2cd4c, Vdr, Cd14, Apod, Dab2, Anxa3, Comp, Faah, Dysf, Esr1, Gas6, Stard10, Ptg2, Pacsin1, Fabp4, Fabp5</i>
GO:0044463	cell projection part	7.21372	0.00073641	<i>Tnfrsf1b, Itkpa, Nefh, Fbxo2, Syng1, Grik5, Nox1, Adrb2, Pfn2, Plek, Ophn1, Ncm1, Dzip1, Kif5c, Ndr4, Trpm6, Trpv4, Ak1, Rapgef4, Ang, Bsn, Fgr, Kirrel3, Flrt3, Aif1, Mapk8ip1, Ank3, Adgrl1, Ccl2, Prkar2b, Apbb1, Dlg2, Dnm1, Rab3a, Eps8, Fscn1, Esr1, Camk2b, Efhc1, Hap1, Gng3, Sntg1, Srcin1, Mylk2, Ptpn5, Ptpn, Pacsin1, Atp6v0a1, Cacna1d, Baiap2, Itga8, Dnaaf1, Eps8l2, Tbc1d10c</i>
GO:0006898	receptor-mediated endocytosis	6.2715	0.0018894	<i>Fcgr1, Mrc1, Msr1, Adrb2, Ophn1, Syt12, Ache, Cd14, Dab2, Dnm1, Calcr1, Pacsin1</i>
GO:0030100	regulation of endocytosis	6.0696	0.00231209	<i>Fcgr1, Fcgr3, C3, Ophn1, Axl, Fgr, Cd14, Ccl2, Sirpa, Dab2, Gas6, Pros1, Ptpn5, Sirpb1a, Pacsin1, Mertk</i>
GO:0030036	actin cytoskeleton organization	5.83643	0.00291924	<i>Lpin1, Itkpa, Iqsec3, Pfn2, Arhgap26, Tnnt2, Plek, Ophn1, Rhou, Arhgef17, Six4, Trpv4, Cdc42ep2, Lurap1, Coro6, Xirp1, Eps8, Fscn1, Ldb3</i>
GO:0019216	regulation of lipid metabolic process	5.81975	0.00296835	<i>C3, Gk, Pik3ip1, Adrb2, Pdk2, Rorc, Dhh, Fgr, Igfbp7, Vdr, Apod, Dab2, Snai1, Esr1, Flt1, Gfi1, Il1a, Klf4, Abcd2, Abcg1</i>
GO:0051764	actin crosslink formation	5.72701	0.00325682	<i>Tnnt2, Eps8, Baiap2</i>
GO:0055099	response to high density lipoprotein particle	5.58822	0.00374168	<i>Abca1, Abcg1</i>
GO:0006897	endocytosis	5.39985	0.00451726	<i>Fcgr1, Mrc1, Mrc2, Msr1, Adrb2, Icam5, Ophn1, Syt12, Arc, Axl, Ache, Cd14, Ccr2, Neur11b, Dab2, Anxa3, Dnm1, Calcr1, Gas6, Pacsin1, Mertk</i>
GO:0032233	positive regulation of actin filament bundle assembly	5.28016	0.00509163	<i>Kiss1r, Pfn2, Plek, Sorbs3, Sdc4, Id1</i>
GO:0030029	actin filament-based process	5.20627	0.00548208	<i>Lpin1, Itkpa, Iqsec3, Pfn2, Arhgap26, Tnnt2, Plek, Ophn1, Rhou, Arhgef17, Six4, Trpv4, Cdc42ep2, Lurap1, Coro6, Xirp1, Apbb1, Eps8, Fscn1, Ldb3</i>
GO:0098858	actin-based cell projection	4.63177	0.00973754	<i>Pdpn, Trpv4, Spn, Slc9a3r2, Apbb1, Cib2, Dcxr, Eps8, Fscn1, Stard10, Srcin1</i>

Gene set	Description	Enrichment score	P-value	Genes
GO:0055094	response to lipoprotein particle	4.39408	0.0123502	<i>Siglec15, Abca1, Abcg1</i>
GO:2000249	regulation of actin cytoskeleton reorganization	4.34517	0.0129693	<i>Rhod, Csf3, Baiap2, Sema3e</i>
GO:0007015	actin filament organization	4.28608	0.0137588	<i>Acta1, Tnnt2, Sorbs3, Rhod, Trpv4, Aif1, Avil, Sirpa, Eps8, Fscn1, Pacsin1, Baiap2</i>
GO:0032231	regulation of actin filament bundle assembly	3.7788	0.0228501	<i>Kiss1r, Pfn2, Plek, Sorbs3, Sdc4, Id1, Kank3</i>
GO:0072583	clathrin-mediated endocytosis	3.53387	0.0291917	<i>Ophn1, Syt12, Dnm1, Pacsin1</i>
GO:0051017	actin filament bundle assembly	3.50194	0.030139	<i>Rhod, Aif1, Eps8, Fscn1, Baiap2</i>
GO:0061572	actin filament bundle organization	3.42535	0.0325378	<i>Rhod, Aif1, Eps8, Fscn1, Baiap2</i>
GO:0031529	ruffle organization	3.41083	0.0330139	<i>Lpin1, Plek, Aif1</i>
GO:0060588	negative regulation of lipoprotein lipid oxidation	3.33074	0.0357665	<i>Apod</i>
GO:0015629	actin cytoskeleton	3.26469	0.0382089	<i>Acta1, Synpo2, Ophn1, Pou6f1, Arc, Bmf, Coro6, Fgr, Hspb7, Fscn1, Srcin1, Baiap2</i>
GO:0008064	regulation of actin polymerization or depolymerization	3.06346	0.0467256	<i>Gm14137, Pfn2, Plek, Cdc42ep2, Avil, Csf3, Eps8, Rasa1, Baiap2, Plekhh2</i>
GO:0005041	low-density lipoprotein receptor activity	3.04659	0.0475205	<i>Olr1, Stab1</i>
GO:0030832	regulation of actin filament length	3.02682	0.0484694	<i>Gm14137, Pfn2, Plek, Cdc42ep2, Avil, Csf3, Eps8, Rasa1, Baiap2, Plekhh2</i>