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RESEARCH ARTICLE

Mobilization of ectopic yolk in *Gallus gallus domesticus*: a novel reverse lipid transport process

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SUMMARY

In many oviparous animals, bursting type atresia of ovarian follicles occurs during the reproductive cycle, resulting in the escape of yolk into the extracellular compartment. In birds, this ectopic yolk is rapidly cleared by an unknown process that involves the appearance of yolk-engorged macrophage-like cells. To study this unique type of lipid transport, we injected young male chickens intra-abdominally with egg yolk. Absorption of egg yolk from the body cavity markedly increased the triacylglyceride-rich fraction (TRL) of plasma lipoproteins and was coincident with increased levels of plasma triacylglycerides (TAGs) but not non-esterified fatty acids (NEFAs). Thus, the transport of yolk lipids from the abdominal cavity appears to occur in lipoproteins and be more similar to the transport of hepatic TAGs to the periphery *via* lipoproteins than to transport of adipose TAGs to the periphery *via* NEFAs released by the action of lipases. When macrophages were exposed to yolk *in vitro*, they quickly phagocytized yolk; however, it is unclear whether this level of phagocytosis contributes significantly to total yolk clearance. Instead, the chicken macrophage may function more as a facilitator of yolk clearance through the modification of yolk lipoproteins and the regulation of the local and systemic immune response to ectopic yolk. Yolk appears to be anti-inflammatory in nature. Yolk did not increase levels of the inflammatory cytokines IL-1, IL-6 and IFNγ either *in vivo* or *in vitro*; in fact, yolk dampened many inflammatory changes caused by lipopolysaccharide (LPS). Conversely, LPS-induced inflammation retarded yolk clearance from the abdominal cavity and plasma TAG levels.

Key words: toll-like receptor, MARCO, CD36, serum amyloid A, apolipoprotein A1, macrophage.

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INTRODUCTION

The movement of lipids between tissues has received tremendous attention because lipids are the most energy dense macronutrient and are also exceptionally difficult to transport because of their hydrophobicity. The molecular details of lipid transport are known for several very important events (Voet and Voet, 2011b). During aerobic exercise, the stored triacylglycerides (TAGs) in adipocytes are delivered to muscle cells *via* non-esterified fatty acids (NEFAs). Following a meal, lipids synthesized *de novo* in hepatocytes are exported to adipocytes *via* very low density lipoproteins (VLDLs) (Voet and Voet, 2011a). These two transport strategies are highly divergent and do not share common mechanisms for mobilization from the cells of origin, transport through the blood or uptake by recipient cells, yet both are capable of quickly moving large quantities of lipid.

Concentrated deposits of lipids are not routinely found outside of cells unless they are moving between tissues *via* blood lipoproteins. But in vertebrates, especially oviparous species, very large masses of lipids are episodically deposited in extracellular locations. This occurs when ovarian large follicles undergo atresia and burst, releasing yolk (Saidapur, 1978), and also when the infundibulum fails to catch ovulating ova, resulting in the yolk bursting in the abdominal cavity (Romagnano, 1996). Normally this excess or misplaced lipid is 'resorbed' and disappears quickly. Though these events occur only during the reproductive season of females and not with the frequency of meal- or exercise-induced lipid transport, they are challenging to understand because the lipid originates outside of cells and is ectopic (i.e. misplaced).

Bursting atresia has been described in numerous avian species (Davis, 1942), though the chicken (*Gallus gallus domesticus*) has been the focus of most mechanistic work. The ovary of a chicken contains 5–10 large yellow yolk-filled hierarchical follicles and hundreds of small white pre-hierarchical follicles. When egg laying ceases because of environmental or nutritional stress, follicular atresia occurs and yolk lipid from the largest follicles is extruded into the abdominal cavity. Within days, the extruded yolk is cleared from the body cavity by a process that has not been well characterized.

There are two major hypotheses for how yolk clearance may occur. In one proposed scenario, yolk is resorbed passively from the body cavity *via* the lymphatic system (Sturkie, 1955). This possibility has not received a lot of attention because of the pervasive belief among biologists that the avian lymphatic system is primitive in nature because of a lack of macroscopically visible lymph nodes. However, chickens possess a well-developed network of blind-ended permeable microvessels that allow for drainage of extracellular fluid directly into the blood *via* the jugular lymphatic sac (Kampmeier, 1969). This could potentially provide a direct avenue for yolk lipoproteins to re-enter the circulation. In the second scenario, yolk is phagocytosed and broken down into primary

components by infiltrating macrophages and/or surrounding cells and then repackaged into novel lipoproteins for secretion into the blood stream. This possibility is supported by the fact that soon after yolk extrusion from follicles, yolk-engorged phagocytes (Nili and Kelly, 1996b) appear in the body cavity. In addition, a novel 20 nm TAG-rich lipoprotein (putatively labeled HDL_R) has previously been detected in the high density lipoprotein plasma fraction of hens undergoing bursting atresia (Walzem et al., 1994; Barron et al., 1999). In this second scenario, it remains unclear whether macrophages, as opposed to local cells and tissues, are the primary mediators of yolk clearance and whether they play a major role in the production of HDL_R. Finally, it is possible that both scenarios are important and that lymphatic flow is responsible for the initial transport and that phagocytes are responsible for removing the remaining lipid that becomes trapped in the extracellular matrix.

Typically, yolk clearance is a highly efficient lipid transport process with hens removing the equivalent of 3–5% of their body mass in egg yolk from the body cavity within 5–10 days (Barron et al., 1999). However, when this process is disrupted by inflammation, fibrinous exudates and coagulated yolk remain in the body cavity (Trampel et al., 2007) – a condition known as egg yolk peritonitis. Inflammation negatively impacts lipid metabolism by macrophages in mammals. Considering that chicken macrophages are potentially playing an active role in yolk clearance and that yolk clearance fails during inflammation, we thought it would be interesting to determine how chicken macrophages respond to egg yolk in the presence and absence of an inflammatory stimulus.

In this study, a tractable laboratory model in which injection of yolk into the abdominal cavity of younger birds that were not otherwise transporting endogenous yolk lipid was employed to examine the changes in the concentration and density distribution of plasma lipoproteins as well as the levels of plasma NEFAs and cholesterol. Additionally, we examined the expression of hepatic apolipoproteins that might be involved in the transport of yolk lipids as well as splenic cytokines in order to determine whether yolk induces an inflammatory response. We decided to use younger birds so that bursting-type atresia could be examined in the absence of non-bursting-type atresia, where the yolk is resorbed directly from an intact follicle without entering the abdominal cavity and without potentially complicating factors, such as reproductive hormones and involuting reproductive organs.

The direct interaction between egg yolk and chicken macrophages was studied *in vitro* by examining cytokine expression and the expression of genes known to be involved in lipid processing and cholesterol efflux (Table 1). Additionally, an inflammatory response was induced by *E. coli*-derived lipopolysaccharide (LPS) in both the *in vivo* and *in vitro* model systems as a positive control for possible inflammatory effects of yolk as well as to inhibit yolk clearance in order to help dissect the underlying mechanism of clearance.

MATERIALS AND METHODS

Chickens

Gallus gallus domesticus (Linnaeus 1758) chicks (1 day old; Ross 308 strain, Cal Cruz Hatcheries, Santa Cruz, CA, USA) were placed in stainless steel box brooders and had free access to water and a nutritionally complete diet (Flock Raiser, Land O' Lakes, Purina Mills, St Louis, MO, USA). Chicks were raised until they were 3 weeks of age, which is prior to sexual maturity (~20 weeks of age). The UC Davis Institution Animal Care and Use Committee approved all experimental procedures.

Egg yolk

Egg yolk was collected from eggs that were laid by white leghorn chickens of W-36 lineage (HyLine, West Des Moines, IA, USA) kept at the UC Davis Avian Research Facility. Laying chickens had free access to feed (Layena, Land O' Lakes, Purina Mills; guaranteed analysis of >16% protein, 2.5% fat, 3.25% calcium and 0.5% phosphorus) and water. Egg yolk was collected only from eggs laid

Table 1. Primer and probe sequences and gene function

Gene	Function	Sequence	Source
IL-1β	Inflammatory cytokine, including regulating lipid metabolism	Probe 5′-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3′ F 5′-GCTCTACATGTCGTGTGTGATGAG-3′ R 5′-TGTCGATGTCCCGCATGA-3′	(Kogut et al., 2003)
IL-6	Inflammatory cytokine, including regulating lipid metabolism	Probe 5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′ F 5′-GCTCGCCGGCTTCGA-3′ R 5′-GGTAGGTCTGAAAGGCGAACAG-3′	(Kogut et al., 2003)
IFNγ	Inflammatory cytokine, including regulating lipid metabolism	Probe 5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′ F 5′-GTGAAGAAGGTGAAAGATATATCATGGA-3′ R 5′-GCTTTGCGCTGGATTCTCA-3′	(Kogut et al., 2003)
28S	Ribosomal RNA	Probe 5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′ F 5′-GGCGAAGCCAGAGGAAACT-3′ R 5′-GACGACCGATTGCACGTC-3′	(Kogut et al., 2003)
ApoA1	Major protein in HDL	F 5′-GCATTCGGGATATGGTGGAC-3′ R 5′-TACTCAAGCTGTTTGCCCACA-3′	(Gentili et al., 2005)
SAA1	Acute phase protein involved in lipid transport	F 5′-GGGCTTCACTTCCACCTGAC-3′ R 5′-AAGCAGATACAGAGCCCACATGGT-3′	(Gentili et al., 2005)
ABCA1	Membrane protein mediating lipid efflux from cells	F 5′-TTACAAAACACGTCCCTGAGGC-3′ R 5′-ACAGCTCGACGAAAGCTCCC-3′	(Gentili et al., 2005)
GAPDH	Glycolytic enzyme	F 5′-AAAGTCGGAGTCAACGGATTTG-3′ R 5′-TGTAAACCATGTAGTTCAGATCGATGA-3′	(Gentili et al., 2005)
SRB1	Scavenger receptor, class B	F 5′-TCATCTCCCACCCTCACTTC-3′ R 5′-GGAGCTTGATGGAGCAGTTC-3′	XM415106

Sequences for the probes and primers (F, forward; R, reverse) used for qRT PCR with references or NLM gene accession numbers.
IL, interleukin; INF, interferon; ApoA1, apolipoprotein A1; SAA1, serum amyloid A1; ABCA1, ATP-binding cassette A1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SRB1, scavenger receptor B1; HDL, high density lipoprotein.

within the 24 h preceding the experiment. Egg yolk was collected aseptically in a laminar flow cell culture cabinet. This was done by thoroughly cleaning the shell with 70% ethanol solution and then disinfecting with a 10% tincture of iodine and allowing the shell to air dry. The egg shells were cracked open and the contents deposited into sterile Petri dishes. A sterile serological pipette was used to collect the egg yolk while avoiding the inclusion of other yolk components.

In vivo yolk reabsorption

At 3 weeks of age the chicks were injected intra-abdominally with 3 ml of one of four solutions: (1) phosphate-buffered saline (PBS); (2) egg yolk diluted 1:1 in PBS; (3) 2 mg kg^{-1} body mass LPS derived from *E. coli* serotype O26:B6 (Sigma-Aldrich, St Louis, MO, USA) dissolved in PBS; and (4) egg yolk and LPS diluted in PBS as described for treatments 2 and 3, respectively. The dose of egg yolk was determined by correcting the average body mass of the birds used in this study by the volume-to-mass ratio of a single egg yolk follicle in a mature laying hen. Particle size analysis (data not shown) confirmed that the dilution of egg yolk in PBS did not alter lipoprotein particle size distribution as compared to native yolk. The LPS dose was chosen based on preliminary experiments that showed 2 mg kg^{-1} body mass was an adequate dose to cause obvious clinical responses such as fever, lethargy, anorexia and sickness behavior, without causing mortality. Prior to injection, the ventral surface of the bird was cleaned with 70% ethanol solution and iodine tincture and allowed to air dry. Birds were then injected with an 18 gauge needle, carefully inserted just cranioventral to the vent, avoiding any viscera. Twelve birds were injected per treatment.

Four birds per treatment were killed by cervical dislocation at 4, 8 and 24 h post-injection. Heparinized blood samples were collected for plasma lipoprotein profile analysis and for measurement of plasma cholesterol, TAG and NEFA concentrations. Plasma was separated by centrifugation at 1000 ***g*** for 15 min and stored at −20°C until needed. The abdominal cavity was inspected for remnants of yolk and then the liver and spleen were collected aseptically and preserved by freeze-clamping in liquid nitrogen. Tissues were stored at −80°C prior to RNA isolation and quantitative reverse transcription-PCR (qRT-PCR) analysis.

Lipoprotein profile analysis

Plasma lipoproteins were labeled with NBD C6-ceramide (Invitrogen, Carlsbad, CA, USA), separated by density gradient ultracentrifugation on a continuous EDTA metal ion complex gradient and then imaged to produce lipoprotein profiles as described elsewhere (Johnson et al., 2005). Briefly, plasma was thawed at room temperature for 30 min and 6 µl was added to 1284 µl of 0.18 mol l^{-1} NaBiEDTA ($C_{10}H_{12}N_2O_8NaBi{\cdot}4H_2O$; TCI America, Portland, OR, USA) and 10 µl of 1 mg ml^{-1} NBD C6-ceramide {6-[(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino] hexanoyl} sphingosine (Invitrogen) in DMSO (EM Science, Darmstadt, Germany) in a total volume of 1300 µl. An 1150 µl aliquot of solution was transferred to a 1.5 ml, 34 mm long, polycarbonate thick-walled ultracentrifugation tube (no. 343778, Beckman-Coulter, Palo Alto, CA, USA). Prepared samples were centrifuged at 5°C and 627,000 ***g*** for 6 h using an Optima TLX ultracentrifuge and TLA 120.2 fixed-angle rotor (Beckman-Coulter). Following centrifugation, tubes were removed and carefully overlaid with ≥200 µl of hexane (EM Science) to eliminate light scattering from the meniscus as this gives false intensity measurements to the TAG-rich lipoprotein (TRL) region.

Imaging and analysis of each sample tube were then carried out according to the methods described previously (Johnson et al., 2005) using the identical instrumental set up. Specific settings for the Microfire camera software were an exposure of 18 ms with a gain of 1.000 and a target intensity of 30% to illuminate the tube prior to image capture as this prevents image saturation. The image of the tube following ultracentrifugation was then converted to a density profile by converting pixel values into intensities, and then plotting those intensities *versus* tube coordinates (0–34 mm) using data analysis software (Origin 7.0, OriginLab, Northampton, MA, USA) to create a lipoprotein density profile. Tube coordinates were related to specific densities through the use of a calibration curve generated using a blank gradient. Peaks for TRL, low density lipoprotein (LDL) and high density lipoprotein (HDL) were identified (Fig. 1). Total lipoprotein intensity and fractional intensities of TRL, LDL and HDL were

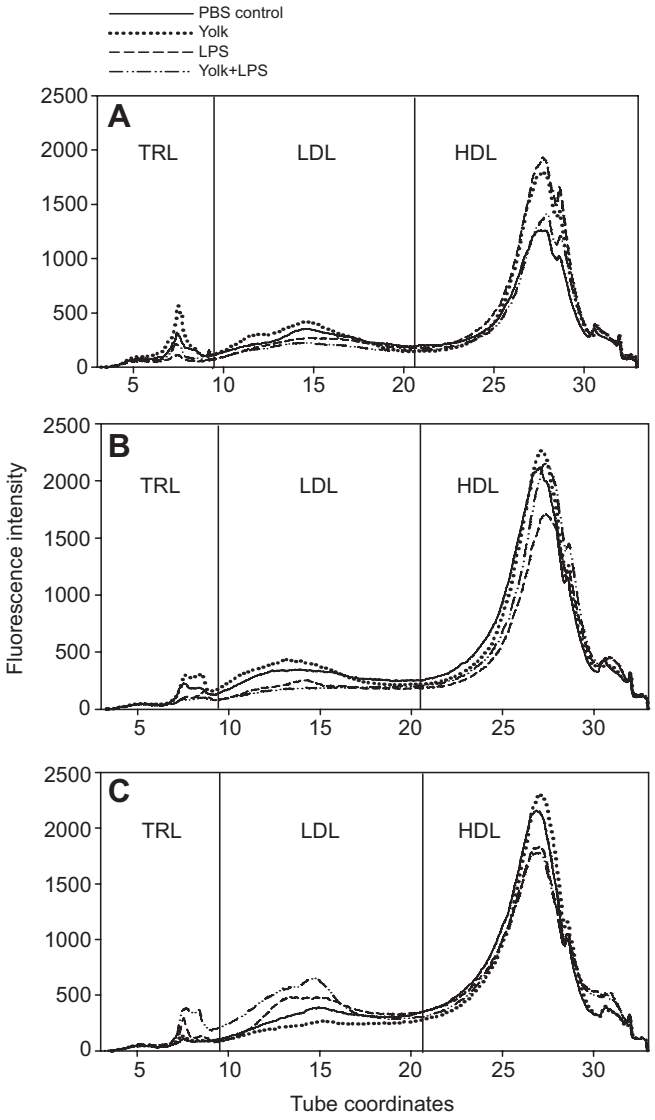

Fig. 1. Plasma lipoprotein profiles. Samples were taken from the control group, egg yolk-injected group, LPS-injected group and egg yolk and LPS co-injected group at 4 h (A), 8 h (B) and 24 h (C) post-injection (*N*=4 each). Plasma lipoproteins were labeled with NBD C6 ceramide and separated by ultracentrifugation on a continuous NaBi EDTA gradient. Traces of fluorescence intensity (arbitrary units) *versus* tube coordinates were converted to plasma lipoprotein profiles. TRL, triacylglyceride-rich lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

determined by measuring the area under the curve of the entire fluorescence trace and each peak, respectively.

Chemical and biochemical measurements

Plasma and cellular cholesterol were measured by Amplex Red Cholesterol Assay (Invitrogen) according to the manufacturer's instructions. Plasma NEFAs and TAGs were measured enzymatically according the manufacturer's instructions (Wako Diagnostics, Richmond, VA, USA). Nitrite concentration in the primary monocyte culture media was measured by Griess assay (Promega, Madison, WI, USA) after 24 h of incubation according to the manufacturer's protocols.

RNA isolation and reverse transcription

RNA extraction was performed using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the frozen tissue sample (liver or spleen) was re-immersed in liquid nitrogen, placed on a paper towel and shattered using a mallet. Approximately 100 mg of tissue was collected using tissue forceps and immersed in 1 ml of Trizol reagent in a microcentrifuge tube. The mixture was homogenized with a polytron power homogenizer until no more particulates could be visualized. Chloroform was then added to the homogenized cell lysate, and phase separation and RNA precipitation were performed according the manufacturer's protocols (Invitrogen).

For *in vitro* studies, tissue culture wells with adherent cells were washed three times with PBS, and then cell-denaturing solution was added to each well (Qiagen RNeasy MiniKit, Valencia, CA, USA). A cell scraper was used to thoroughly mix the cells with the denaturing solution, and the resulting gel was homogenized in a QiaShredder tube (Qiagen) according to the manufacturer's instructions. The sample homogenates were frozen at −80°C for less than 30 days before further RNA extraction was completed using the RNeasy MiniKit (Qiagen) according to the manufacturer's instructions.

The concentration and quality of isolated RNA was assessed spectrophotometrically using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Rochester, NY, USA). Samples with 260 nm:280 nm and 260 nm:230 nm ratios greater than 1.8 were stored at −80°C until needed. Template cDNA was prepared from 400 ng RNA (following DNase treatment) by reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol.

Relative qRT-PCR

Changes in apolipoprotein A1 (ApoA1), serum amyloid A1 (SAA1), scavenger receptor B1 (SRB1) and ATP-binding cassette transporter A1 (ABCA1) in the liver and/or in macrophages were monitored as changes in SYBR Green fluorescence following sample amplification with specific primers. Primer sequences (Table 1) for genes of interest were either derived from published sources or designed using Primer3 Software (Rozen and Skaletsky, 2000) as previously described (Humphrey et al., 2004). The primers were designed to span introns to prevent contamination with genomic DNA and preliminary experiments confirmed that the primer pairs each only produced one product of predicted size. The product sequences (Davis Sequencing, Davis, CA, USA) had at least 99% homology to the genes of interest.

Amplification and detection were performed using a 7500 FAST Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green fluorescence detection of PCR products on 20 µl reaction mixtures containing: 10µl of Fast SYBR Green Mastermix (Invitrogen), 300 nmol l^{-1} each of forward and reverse specific primer and 1 µl of cDNA template. Thermocycler conditions were as follows: initial 10 min denaturation step at 95°C followed by 40 cycles of denaturation (3 s at 95°C) and annealing/extension (30 s at 60°C).

Changes in interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and interferon gamma (IFNγ) gene expression were monitored as changes in Taqman probe fluorescence following sample amplification with specific primers. Primer and probe sequences for Taqman qPCR (Table 1) were derived from a previously published source (Kogut et al., 2003). Quantitative PCR was performed using a 7500 FAST real-time PCR system (Applied Biosystems) on 20 µl reaction mixtures containing 10 µl Taqman FAST Universal PCR Mastermix without UNG (Invitrogen), 1 µl cDNA template, 900 nmol l^{-1} forward and reverse primers and 250 nmol l^{-1} probe.

Relative changes in gene expression were calculated according to the Pflaffl method (Pflaffl, 2001) using LinRegPCR version 5 (Ramakers et al., 2003) to calculate individual specific primer efficiency by linear regression. All cycle threshold fluorescence (Ct) values were calibrated to the average Ct of the PBS-injected control birds or media controls, as appropriate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control for all genes except for interleukin and IFNγ expression where 28S rRNA was used as the endogenous control (the validity of 28S as an endogenous control for cytokines was confirmed in prior publications and 28S functioned well in the Taqman qPCR system) (Kogut et al., 2003).

Monocyte isolation and macrophage culture

Samples of cranial vena cava blood (3 ml) were collected from 3 week old chicks using a 25 gauge needle mounted on a 3 ml heparinized syringe. Each blood sample was carefully layered onto 3 ml of 57% Percoll (Sigma-Aldrich) solution in a 15 ml conical tube (1.71 ml Percoll, 0.33 ml 1 mol l^{-1} NaCl and 0.96 ml ddH_2O). The blood and Percoll were then centrifuged at 1250 ***g*** for 30 min at 4°C. The cells at the interface were collected with a transfer pipette, moved to a new conical tube and suspended in 3 ml of control media consisting of RMPI 1640 with glutamine (Invitrogen), supplemented with 8% fetal bovine serum, 2% chicken serum and 1% penicillin/streptomycin (Invitrogen). The cells were centrifuged again at 400 ***g*** for 4 min at 4°C and the supernatant was removed. The cells were then resuspended in media at a concentration of 5×10^5 cells ml^{-1} and distributed as 1 ml aliquots into a 12-well culture plate. The plate was incubated at 41°C for 10 min in a 5% CO_2 incubator. The media and non-adherent cells were removed and new media added. The plate was incubated for another hour, at which time media and non-adherent cells were removed. Media containing treatments was added to the remaining adherent cells (macrophages).

Chicken macrophages were incubated in one of four treatments: (1) control media; (2) media supplemented with 1% egg yolk; (3) media with 10 µg ml^{-1} LPS; and (4) media with egg yolk and LPS in amounts equal to those in treatments 2 and 3. The dose of egg yolk was chosen based on preliminary experiments that found 1% egg yolk to be ideal for causing microscopically visible lipid engorgement without significant cell death or cell detachment. The dose of LPS was chosen based on preliminary experiments measuring nitric oxide production over time; 10 µg ml^{-1} produced the greatest nitric oxide response without significant cell death. After 4, 12 and 24 h incubation, RNA was isolated from the macrophages and changes in gene expression were measured by qRT-PCR. Cell cholesterol content was measured in 24 h cultures. Briefly, cells were lysed as described previously (Goh et al., 1990) and the cell

cholesterol content was determined enzymatically as described above. The experiment was repeated four times in separate pools of macrophages.

Statistical analysis

For *in vivo* studies, the effects of egg yolk, LPS and time post-injection (2×2×3) on lipoprotein profile, plasma lipid levels and gene expression in the liver and spleen were calculated by 3-way ANOVA using JMP version 8.0 (SAS Institute, Raleigh, NC, USA). For cell culture studies, the effects of egg yolk, LPS and incubation time on relative gene expression and cellular cholesterol content were measured by 3-way ANOVA using JMP. In all cases, *P*-values less than or equal to 0.05 were considered significant.

RESULTS

Egg yolk injection increases TRL plasma fraction and plasma TAGs

To determine how ectopic egg yolk is transported from the body cavity through the plasma, and to determine how inflammation affects normal yolk clearance, plasma lipoprotein density profiles, TAG, NEFA and cholesterol levels were measured 4, 8 and 24 h after intra-abdominal injection with egg yolk and/or LPS (Fig. 1 and Table 2). Birds injected with egg yolk alone had no yolk present in the body cavity 24 h post-injection, indicating successful yolk clearance. However, the body cavities of birds co-injected with LPS still contained visible egg yolk after 24 h, indicating that yolk clearance was greatly diminished. Egg yolk injection increased the TRL plasma fraction compared with that of control birds (mean ± s.e.m. 4.15±0.57% *versus* 2.74±0.29%, respectively), which represents a statistically significant increase in plasma TRL over time (P=0.031), with the highest levels seen 4 h post-injection. Yolk injection also significantly increased total plasma TAG levels over time (P=0.009) without altering plasma cholesterol levels (P=0.648). The fact that no significant increase in plasma cholesterol was detected after egg yolk injection is not surprising because egg yolk does not contain a high amount of cholesterol; the total amount of cholesterol present in a 3 ml yolk injection (~16 mg) (USDA, 2002) is equal to less than 0.5% of the total circulating plasma cholesterol in a 3 week old chick. Egg yolk injection did not alter total lipoprotein fluorescence or the proportion of LDL and HDL fluorescence (P=0.766, P=0.513 and P=0.897, respectively). Yolk injection did not alter plasma NEFA levels (P=0.662). LPS injection significantly attenuated the increase in plasma TAG caused by yolk (interaction, P=0.006), especially at 4 and 8 h post-injection (Fig. 2A), without altering plasma lipoprotein density distribution (total lipoprotein P=0.365, TRL P=0.370, LDL P=0.522 and HDL P=0.370), NEFA (P=0.315) or cholesterol (P=0.153). With the exception of TAGs described above, there was no significant yolk × LPS interaction effect on plasma lipid parameters.

LPS injection alters liver ApoA1 and SAA1 mRNA expression

Liver mRNA expression of ApoA1 and SAA1 was measured 8 h post-injection of yolk and/or LPS (Fig. 2B). Egg yolk by itself did not affect the expression of ApoA1 or SAA1 (P=0.388 and P=0.286, respectively). LPS significantly lowered liver mRNA expression of ApoA1 (P=0.036) and caused a 50-fold increase in SAA1 mRNA expression over control (P<0.001). However, co-injection of egg yolk inhibited LPS-induced increases in liver SAA1 expression as indicated by a significant yolk × LPS interaction (P=0.027).

Egg yolk reduces LPS-induced inflammation

Splenic mRNA expression of IL-1β and IL-6 was measured 4 h post-injection of chicks with egg yolk and/or LPS (Fig. 3A,B). Unlike LPS, egg yolk alone did not significantly increase the expression of either of these pro-inflammatory cytokines. In fact, yolk blunted the increase in IL-1β and IL-6 expression induced by LPS, as evidenced by significant egg yolk × LPS interactions (P=0.043 and P<0.001, respectively). This strong interactive effect was examined in more detail *in vitro*.

Macrophages derived from blood monocytes were incubated *in vitro* with egg yolk and/or LPS, and mRNA expression of IL-1β and IL-6 was measured by qRT-PCR after 4, 12 and 24 h incubation (Fig. 3A,B). IFNγ expression was also measured at three time points, but only showed significant changes due to treatment after 4 h of incubation (Fig. 3C). Nitrite concentration in the media after 24 h of incubation was measured by Griess assay as a marker for nitric oxide production (Fig. 3D). As expected, LPS significantly increased inflammatory mediators, increasing IL-1β, IL6 and IFNγ expression and nitrite concentration (P<0.001, P<0.001, P=0.006 and P<0.001, respectively). When analyzed across all time points, egg yolk treatment did not increase IL-1β, IL-6 or IFNγ mRNA expression and did not affect nitric oxide production. Interestingly, there was a significant egg yolk × LPS interaction effect on IL-1β expression and nitric oxide production (P=0.029 and P=0.002, respectively), indicating that egg yolk significantly reduced LPS-induced increases in IL-1β expression and nitric oxide production. Similar results for

Table 2. Plasma lipid analysis

	Hours post-injection*			Egg yolk		LPS	
Lipid species	4	8	24	–	+	–	+
Total lipoprotein fluorescence (a.u.)	282,903±15,246^{C}	346,880±16,739^{B}	408,808±24,513^{A}	342,45±19,423	349,549±18,321	356,442±13,171	335,952±23,049
% TRL fluorescence	4.45^{A}±0.65	3.31^{A,B}±0.45	2.68^{B}±0.58	2.74±0.29	4.15†±0.57	3.75±0.48	3.18±0.47
% LDL fluorescence	31.7^{A,B}±1.27	28.1^{B}±1.50	34.5^{A}±2.22	32.0±1.38	30.8±1.58	32.0±1.07	30.8±1.80
% HDL fluorescence	63.2±2.00	68.6±1.90	62.8±2.69	64.7±1.75	65.0±2.00	63.8±1.52	66.0±2.15
TAG (mg dl^{-1})	36.74^{B}±7.46	36.67^{B}±7.11	58.22^{A}±6.58	33.08±3.31	53.62†±7.22	54.11±5.97	32.57‡±5.29
NEFA (mg dl^{-1})	0.582^{B}±0.059	0.645^{B}±0.083	1.172^{A}±0.097	0.763±0.077	0.819±0.091	0.847±0.070	0.743±0.098
Cholesterol (mg dl^{-1})	86.32^{B}±6.16	107.1^{A}±5.50	78.25^{B}±3.92	89.15±5.37	92.0±4.5	95.1±3.73	86.05±5.79

TRL, triacylglyceride-rich lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TAG, triacylglycerol; NEFA, non-esterified fatty acid; LPS, lipopolysaccharide.

*With the exception of TAG, there were no statistically significant interactions by 2-way ANOVA so all data are presented as separate main effects (mean ± s.e.m.). The significant interaction between yolk and LPS is illustrated in Fig. 2A. Different uppercase letters indicate time points with significantly different means (P≤0.05). N=4.

†Significant effect due to egg yolk treatment.

‡Significant effect due to LPS treatment.

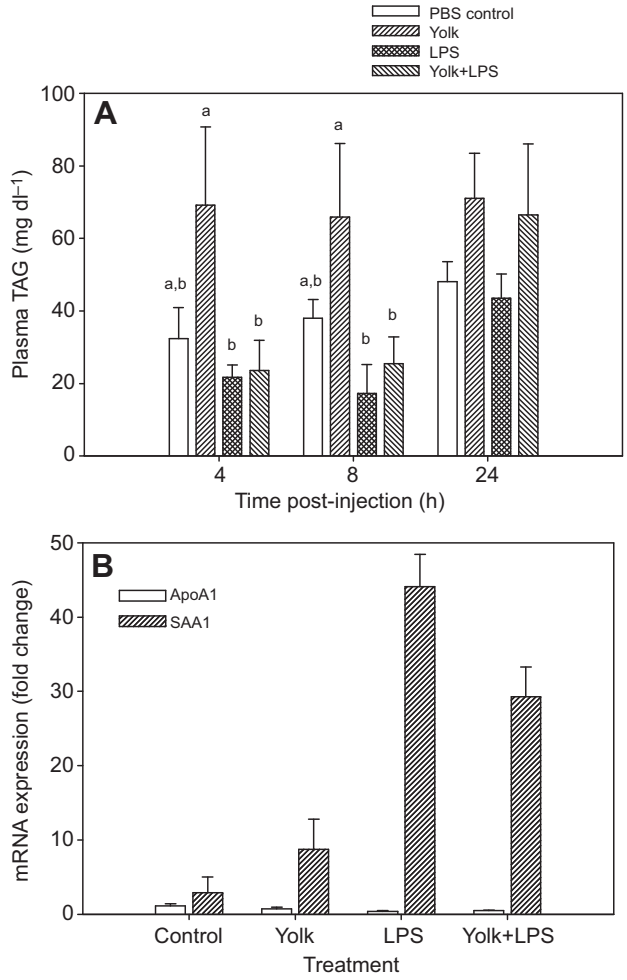

Fig. 2. (A) Plasma triacylglycerol (TAG) concentration, measured enzymatically in the control, egg yolk-injected, LPS-injected and egg yolk and LPS co-injected group at 4, 8 and 24 h post-injection. (B) Relative changes in liver apolipoprotein mRNA expression. ApoA1, apolipoprotein A1; SAA1, serum amyloid A1. Data are means and s.e.m. (N=4). Significantly different means within a time point are indicated by different letters.

nitric oxide production were observed even when LPS treatment preceded egg yolk treatment by at least 1 h (data not shown), indicating that the effect of yolk was not due to a decrease in the interaction of LPS with cell receptors.

LPS increases macrophage mRNA expression of SAA1 but decreases expression of genes involved in reverse cholesterol transport

To determine the effect of inflammation on reverse cholesterol transport of yolk lipid, blood-derived chicken macrophages were incubated *in vitro* with egg yolk and/or LPS, and mRNA expression of ApoA1, SAA1, ABCA1 and SRB1 was measured after 4, 12 and 24 h of incubation (Fig. 4), and cell cholesterol content was determined after 24 h of incubation. ApoA1 mRNA expression remained relatively stable throughout incubation (P=0.347), with egg yolk tending to increase the mRNA expression of ApoA1 (P=0.060) while LPS had no effect on ApoA1 expression (P=0.319). SAA1 expression increased significantly as a result of LPS treatment (P<0.001), while egg yolk had no effect (P=0.672). LPS significantly decreased the expression of ABCA1 and SRB1 (P<0.001 and P<0.001, respectively) and egg yolk had no effect on the expression of either gene (P=0.113 and P=0.324, respectively). Egg yolk increased cell cholesterol content from a mean (±s.e.m.) of 1.83±0.11 to 2.17±0.21 μg ml^{-1} cell lysate (P=0.015) and LPS did not prevent this increase (P=0.872).

DISCUSSION

Birds transport large quantities of yolk during several periods of their life history: *in ovo* and during the early post-hatching period, during the active egg-laying cycle when eggs are being formed, and during follicular atresia (Shackelford and Lebherz, 1983; Rajavashisth et al., 1987; Brown, 1989; Barron et al., 1999). During follicular atresia, large yolk-filled follicles involute and yolk lipid is extruded into the abdominal cavity. To investigate how egg yolk is transported during ovarian atresia, we injected immature chickens intra-abdominally with egg yolk and monitored changes in plasma lipids, hepatic expression of apolipoproteins and splenic expression of inflammatory cytokines. Egg yolk had visibly disappeared within 24 h in those chicks not co-injected with LPS. Absorption of egg yolk from the body cavity markedly increased the TRL fraction of plasma lipoproteins and was coincident with an increase in plasma TAG levels. Plasma NEFA and cholesterol levels were not changed during yolk absorption. Thus, the transport of yolk lipids from the abdominal cavity appears to occur in lipoproteins and be more similar to the transport of hepatic TAG to the periphery *via* VLDL than to transport of adipose TAG to the periphery *via* NEFA released by the action of lipases.

Although plasma HDL tended to be higher in chicks injected with yolk alone than in those injected with PBS at the 4 h time point, we did not observe a statistically significant increase in the amount of this lipoprotein class. This was somewhat surprising as generally when large quantities of yolk are being cleared from the body cavity, as is seen at the end of the laying cycle, a special large diameter (20 nm), TAG-rich, HDL particle (HDL_R) can be transiently found in the plasma (Walzem et al., 1994). This special HDL also appears during yolk absorption in the chick just after hatching and probably plays a role in the movement of lipid from the tissues to the liver (Rajavashisth et al., 1987). However, the amount of yolk that is absorbed during ovarian atresia can exceed 65 ml (Nili and Kelly, 1996a), which is far greater than the amount of egg yolk used in this trial even when differences in body mass of 3 week old chicks and full-grown laying hens are taken into account. Also, during ovarian regression bursting-type and non-bursting-type atresia occur in parallel, with a large quantity of yolk being processed directly within the follicle without entering the body cavity. This follicular yolk, along with large amounts of cholesterol and phospholipid that could arise from remodeling of the oviduct *per se*, may contribute to higher rates of HDL production. Specifically designed experiments would be needed to estimate the contributions from yolk and regressing tissues.

It was previously shown that the concurrence of abdominal inflammation due to *E. coli* impairs the removal of yolk (Gross and Siegel, 1959). Similarly, we found that inflammation induced by LPS impaired yolk clearance from the abdomen. This subjective evaluation is supported by the observation that plasma TAGs were also dampened by LPS, although the acute phase response to LPS was likely accompanied by anorexia (Dantzer et al., 1998) and this may have contributed to decreased plasma TAG. We previously observed that increased liver lipid caused by yolk injection is ameliorated by LPS (data not shown). Thus, inflammation appears to disrupt the normal homeostatic process of yolk clearance in chickens.

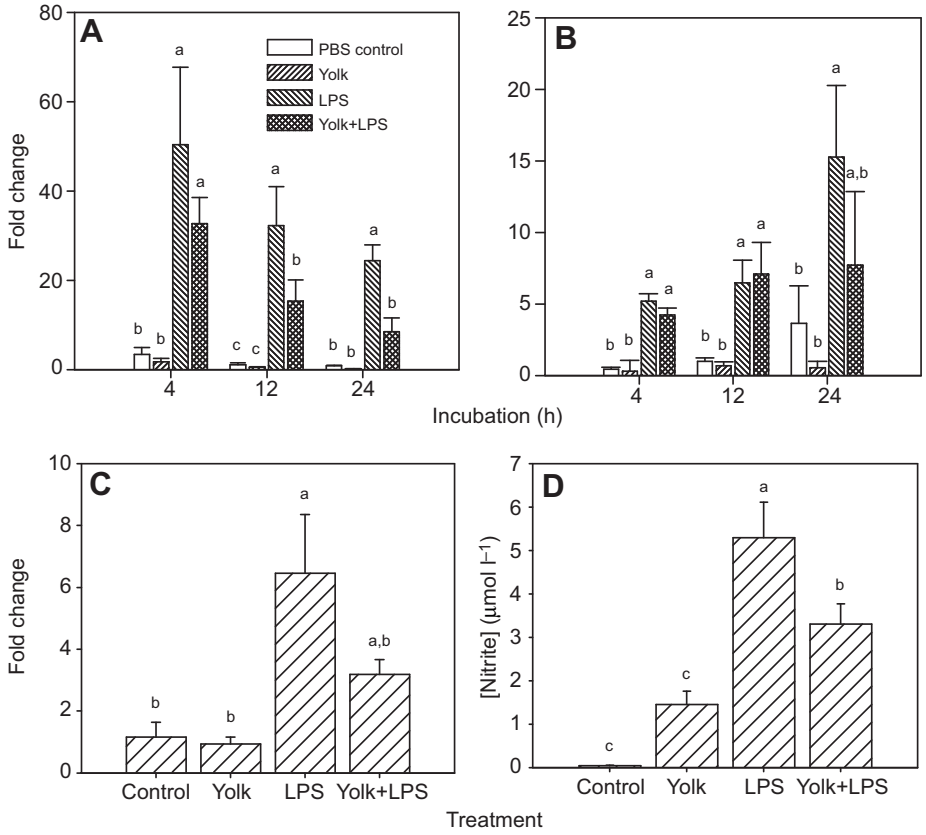

Fig. 3. Inflammatory response of chicken monocytes to egg yolk and/or LPS *in vitro*. Relative changes in interleukin (IL)-1β (A), IL-6 (B) and interferon (IFN)γ (C) mRNA expression and nitric oxide production (D). Data are means and s.e.m. (*N*=4). Significantly different means within a time point are indicated by different letters.

The primary cell that responds to ectopic egg yolk in the body cavity in chickens appears to be the blood-derived monocyte. Chickens have few or no resident abdominal macrophages (Qureshi, 2003; Bliss et al., 2005) and rely on rapid recruitment and proliferation of blood-derived monocytes during an immune response. The same appears to be true during clearance of egg yolk from the body cavity, as yolk-engorged macrophage-like cells appear in the body cavity soon after yolk extrusion. Macrophages cultured *in vitro* remove yolk colloid components from cell culture media and become phenotypically similar to the cells seen *in vivo* (Nili and Kelly, 1996a; Nili and Kelly, 1996b). To determine whether chicken macrophages could play a significant role in the removal of egg yolk lipid from the body cavity, we incubated primary chicken macrophages with yolk *in vitro*. The cells were observed to develop lipid droplets in their cytosol within an hour and became lipid engorged by the end of the 24 h incubation. Indeed, the amount of yolk added needed to be titrated to prevent cell engorgement to the point of 'floating off' the surface of the culture plates. However, even at very low yolk concentrations (1% by volume), there was never a complete clearance of yolk spheres from the media. It is possible that the cultured macrophages lacked the appropriate vehicles such as preformed lipoprotein particles necessary for yolk lipid efflux. This may explain why the expression of genes involved in HDL binding and lipid cholesterol efflux (SRB1 and ABCA1) were only mildly increased following yolk treatment. However, even in perfect conditions, it seems unlikely that the sole purpose of the macrophage response is phagocytosis, repackaging and efflux of yolk lipid. If we were to assume that *in vitro* experimental conditions represented one round of yolk uptake by the macrophages without efflux, we can approximate the contribution of macrophages to yolk clearance *in vivo*. *In vitro*, at the beginning of incubation, each culture well contained 5×10^5 macrophages and 30 µl of egg yolk, and if we were to generously assume that all yolk was removed then each cell would have to remove 60 pl of yolk from the media. When this calculation is applied to the *in vivo* model, where 1.5 ml of injected yolk was cleared from the body cavity in 24 h, a minimum of 2.5×10^{10} macrophages would be required to clear all yolk in one round of yolk uptake. Assuming a 0.75 blood volume to body mass ratio (Kiepper, 2009) and an average blood monocyte count of 1300 cells µl^{-1} blood (Seliger et al., 2012), the average 500 g chicken used in this experiment had ~5×10^7 total monocytes available for recruitment from the blood into the body cavity, or three orders of magnitude fewer monocytes than would be required for effective yolk clearance. Conversely, if every available blood monocyte were to participate in yolk clearance then each cell would have to perform at least 1000 rounds of yolk uptake and efflux. Therefore, considering that yolk clearance coincides with an increase in the TRL plasma lipoprotein class, it seems most likely that the majority of yolk lipid flows with interstitial fluids to be passively absorbed by lymphatic vessels and delivered directly into the plasma without repackaging by macrophages or local cells and tissues.

So, if macrophages do not play a major role in yolk uptake, repackaging and efflux, their major function in yolk clearance is called into question. One major function appears to be removal of yolk that is trapped between interstitial membranes and around air sacks. Histological examination of tissues from birds undergoing atresia shows that the yolk-engorged phagocytes appear to be primarily associated with the interstitium, air sacs and ovarian tissues (Nili and Kelly, 1996a).

Another potential function is the modification of yolk lipoproteins to facilitate liver-mediated clearance. Macrophages secrete cholesteryl ester transfer protein (CETP) in response to ApoA1 (Niculescu et al., 2011). CETP transfers cholesteryl esters from HDL to LDL and VLDL particles. These cholesteryl ester-enriched

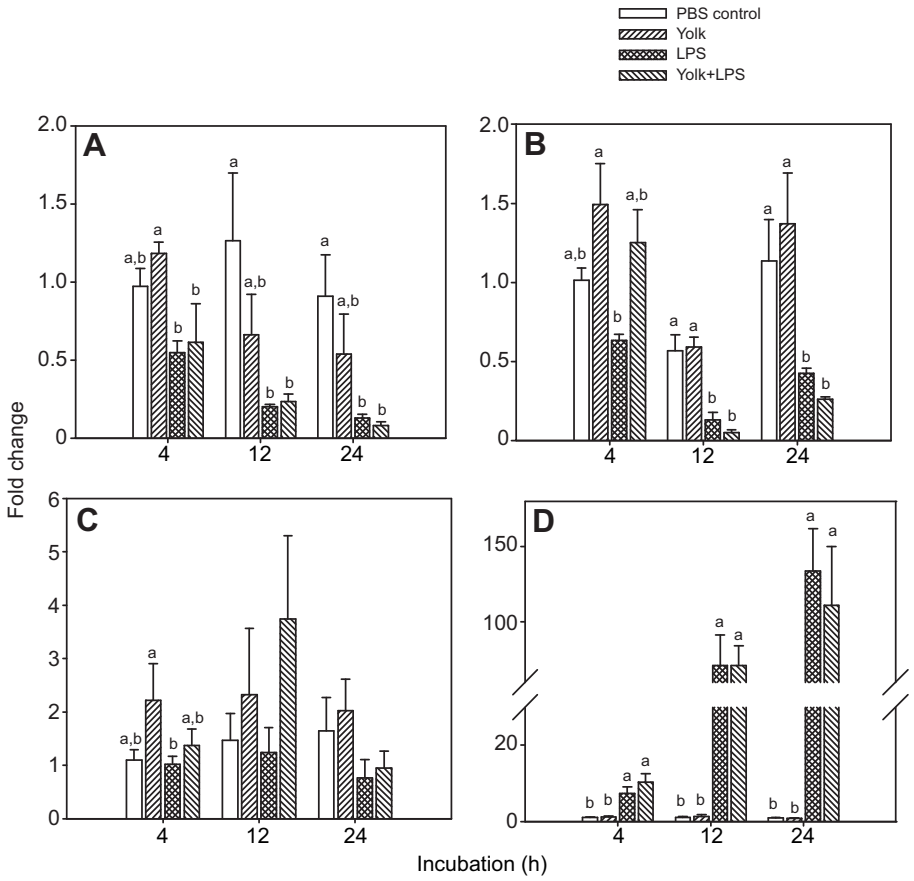

Fig. 4. Relative changes in chicken monocyte cholesterol efflux gene and apolipoprotein expression: scavenger receptor B1 (SRB1) (A), ATP-binding cassette A1 (ABCA1) (B), apolipoprotein A1 (ApoA1) (C) and serum amyloid A1 (SAA1) (D). Data are means and s.e.m. (*N*=4). Significantly different means within a time point are indicated by different letters.

particles are good substrates for selective cholesteryl ester uptake *via* hepatic lipase or can directly bind hepatic LDL receptors to allow mass clearance from the plasma (Barter et al., 2003). In this experiment, we found that chicken macrophages expressed ApoA1 mRNA and it is possible that, in the chicken, ApoA1 expression may cause autocrine stimulation of CETP secretion.

ApoA1 is the only surface apolipoprotein found on functional HDL in chickens. ApoA1 is also found on the surface of mammalian HDL in addition to ApoE. Although previous studies have indicated that ApoA1 is expressed in a variety of tissues in the chicken, including hepatocytes, intestinal cells, adipocytes and myocytes (Blue et al., 1982; Shackelford and Lebherz, 1983; Rajavashisth et al., 1987; Brown, 1989), this is the first report showing ApoA1 expression in macrophages. Intriguingly, as ApoA1 is the only apolipoprotein required for HDL formation in chickens, the possibility that chicken macrophages have the unique ability to produce HDL precursors in order to mediate lipid efflux cannot be ruled out. In fact, Gentili and colleagues (Gentili et al., 2005) found that ApoA1 plays a key role in cholesterol efflux from chicken chondrocytes during homeostasis, while another apolipoprotein, SAA1, became the primary apolipoprotein for lipid efflux from chondrocytes during an inflammatory response. They also found that mRNA expression of ApoA1 and SAA1 reflected protein levels, and that production of ApoA1 by chondrocytes was a sufficient indicator of HDL production. Similar to what was reported in chondrocytes (Gentili et al., 2005), we demonstrated that macrophages also increase SAA1 expression in response to inflammation, which may indicate a change in the nature of effluxed HDL particles. During an acute phase response in mammals, up to 80% of HDL ApoA1 is replaced by SAA1 as the result of a switch from ApoA1 to SAA1 production by the liver (Kontush and Chapman, 2006). The replacement of ApoA1 by SAA1 renders HDL dysfunctional by decreasing its anti-oxidant, anti-inflammatory and cholesterol efflux potential (Kontush and Chapman, 2006). The role of HDL particles that are rich in SAA1 appears to be the delivery of lipid to immune cells, especially macrophages, during an immune response. This highly evolutionarily conserved acute phase reaction appears to be important in the clearance of pathogens; however, it severely limits macrophage-mediated lipid efflux in mammals (Kontush and Chapman, 2006). A change in HDL functionality may at least be partially responsible for the failed yolk clearance seen when yolk and LPS were co-injected into birds.

An additional function of macrophages is responding to any foreign material present in the body cavity in order to determine the nature of that material and then mediate the appropriate local and systemic response to that material. Egg yolk appears to be anti-inflammatory in nature. Co-injection of egg yolk and LPS limited the extent of the acute phase response *in vivo*, and when macrophages were incubated *in vitro* with both egg yolk and LPS, the inflammatory response was reduced. Egg yolk dampened LPS-induced induction of IL-1β expression and decreased LPS-induced nitric oxide production. Egg yolk also appeared to reduce LPS-mediated IL-6 expression (late in the response) and IFNγ expression. Initially, we believed these changes reflected binding of LPS by egg yolk lipoproteins, a known anti-inflammatory function of lipoproteins such as HDL (Yvan-Charvet et al., 2010). However, LPS-induced expression of IL-6 remained high for the early time points despite egg yolk treatment, and the addition of egg yolk to the cell culture media 1 h after the addition of LPS yielded similar decreases in nitric oxide production to those seen when yolk and

LPS were added together. Other explanations for the anti-inflammatory effects of egg yolk include the presence of anti-inflammatory yolk components such as polyunsaturated fatty acids (PUFAs), which are known to decrease signaling *via* the pro-inflammatory nuclear factor kappa B (NFκB) in a Toll-like receptor (TLR4)-dependent manner (Lee et al., 2003), and antioxidants such as carotenoids and vitamin E. Finally, it is possible, given that egg yolk appeared to increase ApoA1 and ABCA1 expression, that yolk uptake leads to the activation of liver X receptors (LXRs). Upon binding cholesterol metabolites, LXRs downregulate NFκB-induced increases in IL-1β and IL-6 expression and upregulate genes involved in cholesterol efflux such as ABCA1 and ApoA1 (Castrillo et al., 2003; Zelcer and Tontonoz, 2006). Conversely, LPS inhibits LXR-mediated increases in SRB1, ABCA1 and ApoA1 expression (Lakomy et al., 2009), which is also in agreement with what was found in the current study.

Bulk transport of lipid poses a great challenge to multiple species because of its energy density and hydrophobic nature. Even greater problems occur when that lipid needs to be transported from the extracellular space instead of from the digestive tract, adipose tissue or hepatocytes. Birds have evolved a highly efficient lipid transport process for removal of lipid-rich ectopic yolk from the body cavity. Based on the data from this study, we posit that macrophages could play a multifaceted role in yolk clearance, potentially clearing 'trapped' yolk, modifying yolk lipoproteins to facilitate plasma clearance and managing the immune response to foreign material in the abdominal cavity. We also found that despite the remarkable efficiency of ectopic yolk transport and the apparently anti-inflammatory nature of yolk, yolk lipid clearance is highly sensitive to inflammation, with LPS preventing the removal of yolk from the body cavity. Future experiments should explore the influence of sex hormones on yolk clearance, and determine whether and how chicken macrophages modify yolk lipoproteins and whether chicken macrophages are capable of producing HDL precursors.

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette A1
ApoA1	apolipoprotein A1
IL-1β	interleukin 1 beta
IL-6	interleukin 6
LPS	lipopolysaccharide
LXR	liver X receptor
NEFA	non-esterified fatty acid
NFκB	nuclear factor kappa B
SAA1	serum amyloid A1
SRB1	scavenger receptor B-1
TAG	triacylglycerol

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AUTHOR CONTRIBUTIONS

I.C., R.L.W. and K.C.K. conceived and designed the study and interpreted the data. I.C. executed the study, conducted the statistics and wrote the manuscript, with revisions by R.L.W. and K.C.K. Lipoprotein profile analysis and interpretation were provided by C.L., R.L.W. and R.D.M.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

Barron, L. G., Walzem, R. L. and Hansen, R. J. (1999). Plasma lipoprotein changes in hens (*Gallus domesticus*) during an induced molt. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **123**, 9-16.

Barter, P. J., Brewer, H. B., Jr, Chapman, M. J., Hennekens, C. H., Rader, D. J. and Tall, A. R. (2003). Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **23**, 160-167.

Bliss, T. W., Dohms, J. E., Emara, M. G. and Keeler, C. L., Jr (2005). Gene expression profiling of avian macrophage activation. *Vet. Immunol. Immunopathol.* **105**, 289-299.

Blue, M.-L., Ostapchuk, P., Gordon, J. S. and Williams, D. L. (1982). Synthesis of apolipoprotein AI by peripheral tissues of the rooster. A possible mechanism of cellular cholesterol efflux. *J. Biol. Chem.* **257**, 11151-11159.

Brown, E. M. (1989). Effects of phospholipids on the conformation of chicken high density lipoprotein. *Poult. Sci.* **68**, 399-407.

Castrillo, A., Joseph, S. B., Vaidya, S. A., Haberland, M., Fogelman, A. M., Cheng, G. and Tontonoz, P. (2003). Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism. *Mol. Cell* **12**, 805-816.

Dantzer, R., Bluthé, R. M., Gheusi, G., Cremona, S., Layé, S., Parnet, P. and Kelley, K. W. (1998). Molecular basis of sickness behavior. *Ann. New York Acad. Sci.* **856**, 132-138.

Davis, D. E. (1942). The regression of the avian post-ovulatory follicle. *Anat. Rec.* **82**, 297-307.

Gentili, C., Tutolo, G., Pianezzi, A., Cancedda, R. and Descalzi Cancedda, F. (2005). Cholesterol secretion and homeostasis in chondrocytes: a liver X receptor and retinoid X receptor heterodimer mediates apolipoprotein A1 expression. *Matrix Biol.* **24**, 35-44.

Goh, E. H., Krauth, D. K. and Colles, S. M. (1990). Analysis of cholesterol and desmosterol in cultured cells without organic solvent extraction. *Lipids* **25**, 738-741.

Gross, W. B. and Siegel, P. B. (1959). Coliform peritonitis of chickens. *Avian Dis.* **3**, 370-373.

Humphrey, B. D., Stephensen, C. B., Calvert, C. C. and Klasing, K. C. (2004). Glucose and cationic amino acid transporter expression in growing chickens (*Gallus gallus domesticus*). *Comp. Biochem. Physiol.* **138A**, 515-525.

Johnson, J. D., Bell, N. J., Donahoe, E. L. and Macfarlane, R. D. (2005). Metal ion complexes of EDTA as solutes for density gradient ultracentrifugation: influence of metal ions. *Anal. Chem.* **77**, 7054-7061.

Kampmeier, O. F. (1969). *Evolution and Comparative Morphology of The Lymphatic System*. Springfield, IL: Charles C. Thomas.

Kiepper, B. (2009). *Processing Tip… Back to Basics: Revisiting Blood Collection* (May 2009). Athens, GA: University of Georgia Cooperative Extension Service, College of Agricultural and Environmental Sciences.

Kogut, M. H., Rothwell, L. and Kaiser, P. (2003). Differential regulation of cytokine gene expression by avian heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized *Salmonella enteritidis*. *J. Interferon Cytokine Res.* **23**, 319-327.

Kontush, A. and Chapman, M. J. (2006). Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacol. Rev.* **58**, 342-374.

Lakomy, D., Rébé, C., Sberna, A.-L., Masson, D., Gautier, T., Chevriaux, A., Raveneau, M., Ogier, N., Nguyen, A. T., Gambert, P. et al. (2009). Liver X receptor-mediated induction of cholesteryl ester transfer protein expression is selectively impaired in inflammatory macrophages. *Arterioscler. Thromb. Vasc. Biol.* **29**, 1923-1929.

Lee, J. Y., Ye, J., Gao, Z., Youn, H. S., Lee, W. H., Zhao, L., Sizemore, N. and Hwang, D. H. (2003). Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J. Biol. Chem.* **278**, 37041-37051.

Niculescu, L. S., Robciuc, M. R., Sanda, G. M. and Sima, A. V. (2011). Apolipoprotein A-I stimulates cholesteryl ester transfer protein and apolipoprotein E secretion from lipid-loaded macrophages; the role of NF-κB and PKA signaling pathways. *Biochem. Biophys. Res. Commun.* **415**, 497-502.

Nili, H. and Kelly, W. R. (1996a). Form and function of lacunae in the ovary of the laying hen. *Anat. Rec.* **244**, 165-174.

Nili, H. and Kelly, W. R. (1996b). In vitro responses of avian monocytes to homologous yolk. *Anat. Rec.* **246**, 458-464.

Pfaffl, M. (2001). A new mathematical model for relative quantification in real-time PCR. *Nucleic Acids Res.* **29**, e45.

Qureshi, M. A. (2003). Avian macrophage and immune response: an overview. *Poult. Sci.* **82**, 691-698.

Rajavashisth, T. B., Dawson, P. A., Williams, D. L., Shackleford, J. E., Lebherz, H. and Lusis, A. J. (1987). Structure, evolution, and regulation of chicken apolipoprotein A-I. *J. Biol. Chem.* **262**, 7058-7065.

Ramakers, C., Ruijter, J. M., Deprez, R. H. L. and Moorman, A. F. M. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339**, 62-66.

Romagnano, A. (1996). Avian obstetrics. *Seminars in Avian and Exotic Pet Medicine* **5**, 180-188.

Rozen, S. and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Protocols: Methods in Molecular Biology* (ed. S. Krawetz and S. Misener). Totowa, NJ: Humana Press.

Saidapur, S. K. (1978). Follicular atresia in the ovaries of nonmammalian vertebrates. *Int. Rev. Cytol.* **54**, 225-244.

Seliger, C., Schaerer, B., Kohn, M., Pendl, H., Weigend, S., Kaspers, B. and Härtle, S. (2012). A rapid high-precision flow cytometry based technique for total white blood cell counting in chickens. *Vet. Immunol. Immunopathol.* **145**, 86-99.

Shackelford, J. E. and Lebherz, H. G. (1983). Regulation of apolipoprotein A1 synthesis in avian muscles. *J. Biol. Chem.* **258**, 14829-14833.

Sturkie, P. (1955). Absorption of egg yolk in the body cavity of the hen. *Poult. Sci.* **34**, 736-737.

Trampel, D. W., Wannemuehler, Y. and Nolan, L. K. (2007). Characterization of *Escherichia coli* isolates from peritonitis lesions in commercial laying hens. *Avian Dis.* **51**, 840-844.

USDA (2002). Nutrient data 01125, egg, yolk, raw, fresh. In *National Food and Nutrient Analysis Program Wave 6d*, Vol. 2012. Beltsville, MD: USDA.

Voet, D. and Voet, J. (2011a). Lipid metabolism. In *Biochemistry*, pp. 990-1018. New York, NY: John Wiley and Sons.

Voet, D. and Voet, J. (2011b). Lipids and membranes. In *Biochemistry*, pp. 386-466. New York, NY: John Wiley and Sons.

Walzem, R. L., Davis, P. A. and Hansen, R. J. (1994). Overfeeding increases very low density lipoprotein diameter and causes the appearance of a unique lipoprotein particle in association with failed yolk deposition. *J. Lipid Res.* **35**, 1354-1366.

Yvan-Charvet, L., Kling, J., Pagler, T., Li, H., Hubbard, B., Fisher, T., Sparrow, C. P., Taggart, A. K. and Tall, A. R. (2010). Cholesterol efflux potential and antiinflammatory properties of high-density lipoprotein after treatment with niacin or anacetrapib. *Atheroscler. Thromb. Vasc. Biol.* **30**, 1430-1438.

Zelcer, N. and Tontonoz, P. (2006). Liver X receptors as integrators of metabolic and inflammatory signaling. *J. Clin. Invest.* **116**, 607-614.