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*Stem Cell Research & Therapy* 2013, **4**:135 doi:10.1186/scrt346

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**ISSN** 1757-6512

**Article type** Research

**Submission date** 19 March 2013

**Acceptance date** 23 October 2013

**Publication date** 1 November 2013

**Article URL** <http://stemcellres.com/content/4/6/135>

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# Potential beneficial effects of ixmyelocel-T in the treatment of atherosclerotic diseases

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## Abstract

### Introduction

Advanced atherosclerotic lesions are characterized by lipid accumulation, inflammation, and defective efferocytosis. An ideal therapy should address all aspects of this multifactorial disease. Ixmyelocel-T therapy, an expanded autologous multicellular therapy showing clinical promise in the treatment of diseases associated with advanced atherosclerosis, includes a novel population of M2-like macrophages. Here, we examine the macrophages of ixmyelocel-T and determine their ability to influx modified cholesterol in an atheroprotective manner, maintaining cholesterol homeostasis and preventing cellular dysfunction and death, ultimately promoting reverse cholesterol efflux.

### Methods

Approximately 50 mL of whole bone marrow was obtained from healthy donors and shipped overnight. Bone marrow mononuclear cells (BMMNCs) were produced using density gradient separation and cultured for approximately 12 days to generate ixmyelocel-T. CD14<sup>+</sup> cells were isolated from ixmyelocel-T via positive selection for analysis. Ixmyelocel-T and human leukemia monocyte (THP-1) cells were loaded with acetylated low-density lipoprotein (Ac-LDL) for analysis. Flow cytometry and immunofluorescence were used to examine Ac-LDL uptake, expression of cytokines was analyzed by ELISA, and quantitative real-time PCR was used to analyze expression of cholesterol transport genes. Both the *in vitro* cholesterol efflux assay and *in vivo* reverse cholesterol transport assay were employed to examine cholesterol transport.

## Results

Ixmyelocel-T macrophages take up acetylated low-density lipoprotein, and express the scavenger receptors CD36 and scavenger receptor-B1 (SR-B1). Ixmyelocel-T did not become apoptotic or pro-inflammatory after lipid loading. The cholesterol transporter genes ABAC1 and ABCG1 were both statistically significantly upregulated when ixmyelocel-T macrophages were loaded with cholesterol. Ixmyelocel-T also exhibited enhanced apolipoprotein A-I (ApoAI)-mediated cholesterol efflux. In addition, *in vivo* reverse cholesterol transport assay demonstrated that ixmyelocel-T was able to efflux cholesterol in this model.

## Conclusions

Ixmyelocel-T macrophages influx modified cholesterol, remain anti-inflammatory in the face of lipid loading and inflammatory challenge, and display enhanced cholesterol efflux capabilities. These combined features suggest that this autologous multicellular therapy may exert beneficial effects in atherosclerotic diseases.

## Introduction

Atherosclerosis, a chronic inflammatory disease of the vessel wall, is a leading cause of death in developed countries [1]. Accumulation of lipid-loaded macrophage foam cells is a central feature in the formation of atherosclerosis. Macrophages, the most abundant cell type in atherosclerotic lesions, accumulate lipids, secrete inflammatory cytokines, and participate in all phases of atherosclerosis [2]. Maintaining macrophage cholesterol homeostasis is essential in preventing the pathogenesis of atherosclerosis [3]. Macrophage scavenger receptors, such as scavenger receptor (SR)-A and CD36, mediate the unregulated uptake of modified LDL lipoproteins, which leads to foam cell formation [3-5]. Cholesterol efflux, which removes excess cholesterol from macrophages, is believed to be the major process involved in the regression of atherosclerotic lesions [6,7]. The net flux, or influx and efflux, of modified cholesterol plays a key role in maintaining cholesterol homeostasis in macrophages [8]. An imbalance between cholesterol uptake by scavenger receptors and efflux in macrophages is widely recognized as the underlying mechanism leading to progression of atherosclerosis [3,9].

Macrophage cholesterol efflux is considered to be protective against the development of atherosclerosis [10]. Free or unesterified cholesterol (FC) is toxic to macrophages and can lead to apoptosis [11]. To prevent cell death, cholesterol is stored as cholesterol esters (CE) within macrophage foam cells and effluxed as FC. Cholesterol efflux is mediated by the ATP-binding cassette transporters ABCA1 and ABCG1 [3]. ABCA1 regulates cholesterol efflux to lipid-free apolipoprotein A-I (apoA-I), and ABCG1 regulates cholesterol efflux to high-density lipoprotein (HDL) [12,13]. Both ABCA1 and ABCG1 prevent cholesterol accumulation and play an important role in maintaining macrophage cholesterol homeostasis. Excess ingested cholesterol is esterified by acyl-CoA:cholesterol acyl-transferase 1 (ACAT-1) and stored as lipid droplets in macrophages [3]. Cholesterol ester hydrolase (CEH) releases free cholesterol from stored CE, allowing it to be removed from macrophages through cholesterol efflux [5]. Studies have shown that macrophages with high CEH activity accumulate fewer CE. Promoting macrophage cholesterol efflux could prevent progression or induce regression of atherosclerosis [11].

Macrophages are a diverse population of cells that adapt and respond to a variety of signals, including cytokines and microbial products [14]. Classically activated macrophages, or M1, produce pro-inflammatory cytokines and kill microorganisms [14], while alternatively activated macrophages, or M2, regulate the inflammatory response by producing anti-inflammatory cytokines, scavenging debris, and promoting tissue repair [14-18]. Both populations are considered extremes of a wide variety of macrophage phenotypes [19]. Atherosclerotic plaques have been found to contain a mixture of M1 and M2 macrophages [20,21]. Recent studies have suggested that M1 macrophages are dominant in both initial and developing atherosclerotic lesions, whereas M2 macrophages are associated with lesion regression [20,22,23]. It is thought that M2 macrophages may help prevent lesion progression by secreting anti-inflammatory cytokines, such as interleukin (IL)-10, and efficiently removing apoptotic cells [20].

Ixmyelocel-T is an expanded autologous multicellular therapy containing a combination of cell types derived from cultured bone marrow mononuclear cells [24]. Ledford et al. found that ixmyelocel-T contains a population of unique M2-like macrophages (Ledford KJ, Parrish C, Zeigler F, Bartel RL: Ixmyelocel-T, an expanded multicellular therapy, contains a population of alternatively activated macrophages, Forthcoming) that are characterized by secretion of the anti-inflammatory cytokines IL-10 and IL-1ra, with minimal secretion of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF $\alpha$ ) and IL-12 after inflammatory challenge (Ledford KJ, Parrish C, Zeigler F, Bartel RL: Ixmyelocel-T, an expanded multicellular therapy, contains a population of alternatively activated macrophages, Forthcoming). Furthermore, these macrophages have demonstrated efferocytosis (ie, efficient removal of apoptotic cells) (Ledford KJ, Parrish C, Zeigler F, Bartel RL: Ixmyelocel-T, an expanded multicellular therapy, contains a population of alternatively activated macrophages, Forthcoming). Both of these characteristics have been hypothesized to have beneficial effects in the treatment of atherosclerosis: IL-10 inhibits the secretion of inflammatory cytokines from other cells [20] and efferocytosis is considered atheroprotective [20]. In fact, defective efferocytosis has been shown to promote atherosclerosis [25]. The goal of this investigation was to further examine ixmyelocel-T macrophages and determine the ability of these cells to influx/efflux modified cholesterol in an atheroprotective manner, by maintaining cholesterol homeostasis and preventing cellular dysfunction and death, ultimately promoting reverse cholesterol efflux. In this study, THP-1 macrophages were utilized as a control in these investigations. THP-1 cells are commonly used as a model to delineate macrophage gene expression and functions associated with modified cholesterol loading and have been established as an effective model to study the effects of cholesterol homeostasis [26-29].

## **Methods**

### **Cell culture**

For the generation of ixmyelocel-T commercially available bone marrow aspirates (Lonza, MD, USA) were obtained from healthy donors under informed consent. A small amount (~50 mL) of whole bone marrow was obtained through needle aspiration of the posterior iliac crest, and stored in heparinized tubes. The mononuclear cell fraction was obtained through an automated, closed-system, Ficoll-based density gradient centrifugation separation process. The isolated mononuclear cells were then transferred to a sterile, single-use cell cassette [30]. This proprietary system controlled temperature, culture medium exchange, and gas exchange during the culture period. After approximately 12 days, the cells were washed and harvested

from the cassette by a multistep, automated process. The cells were then used for experimental study.

THP-1 cells were obtained from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 (ATCC) containing 10% fetal bovine serum and 2 mM L-Glutamine according to the manufacturer's instructions. THP-1 monocytes were differentiated into macrophages using 200 nM phorbol myristate acetate (InvivoGen, San Diego, CA, USA) for 3 days, after which they were used in the following experiments.

### **CD14+ cell purification**

CD14+ cells were isolated from ixmyelocel-T by positive selection using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's directions. After positive selection, the CD14+ ixmyelocel-T macrophages were transferred to 6-well culture plates for subsequent experiments.

### **Dil-Ac-LDL immunostaining**

For fluorescent imaging CD14+ ixmyelocel-T and THP-1 macrophages were plated in glass chamber slides and allowed to adhere. Cells were loaded with 10 µg/mL Dil-Ac-LDL in serum-free media containing 0.2% bovine serum albumin (BSA; fatty acid-free; Sigma-Aldrich, St. Louis, MO, USA). After 24 hours, the cells were washed with phosphate buffered saline (PBS) and fixed in 10% formalin for 10 minutes. Counterstaining was performed with DAPI to visualize nuclei. Fluorescent images were visualized using a Nikon Eclipse 80i (Nikon, Melville, NY, USA) equipped with an EXi Aqua Bio-Imaging Microscopy Camera (Q Imaging, Surrey, BC, Canada). For flow cytometry quantification of ac-LDL uptake ixmyelocel-T and THP-1 macrophages were loaded with 10 µg/mL Dil-Ac-LDL in serum-free media. DiI-AcLDL uptake was analyzed by flow cytometry with at least 10,000 events acquired for each sample. DiI-AcLDL uptake was quantified by mean fluorescent intensity, which was calculated by dividing the value of positive gated events by the value of the negative population.

### **Cytokine secretion**

Enzyme-linked immunosorbent assay kits were used to determine the concentrations of IL-1β, TNFα, IL-6, and monocyte chemoattractant protein (MCP)-1 (R&D Systems, Minneapolis, MN, USA). Briefly, CD14+ ixmyelocel-T and THP-1 macrophages were loaded with 50 µg/mL acetylated low-density lipoprotein (Ac-LDL; Fisher Scientific, Pittsburg, PA, USA) for 24 hours in serum-free media containing 0.2% BSA (fatty acid-free). After 24 hours, the cells were washed with PBS and treated with lipopolysaccharide (LPS; 0.1 µg/mL) overnight. Supernatants were then collected and assayed following manufacturer's directions. For cytokine analysis, the values were normalized to cell number.

### **Apoptosis assay**

Apoptosis was measured in both ixmyelocel-T and THP-1 macrophages loaded with Ac-LDL. Briefly, cells were seeded in 96-well plates and loaded with 100 µg/mL Ac-LDL for 24 hours in serum-free media containing 0.2% BSA (fatty acid-free). The concentration of Ac-LDL used to induce apoptosis was based on our preliminary dose-finding study, which

demonstrated that loading with 100 µg/mL Ac-LDL induced apoptosis in THP-1 macrophages. Induction of apoptosis was examined by measuring the caspase-3 and caspase-7 activity using the Caspase-Glo® 3/7 Assay (Promega, Switzerland). Luminescence units were measured using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA) and values were expressed as a percentage relative to the values obtained from the control group (THP-1 macrophages).

### **Cholesterol efflux assay**

Ixmyelocel-T and THP-1 macrophages were plated at  $0.5 \times 10^6$  cells/well and labeled with  $^3\text{H}$ -cholesterol for 24 hours, and allowed to equilibrate overnight. Cholesterol efflux was measured using 20 µg/mL apoA-I and 20 µg/mL HDL<sub>3</sub> in acceptor media after 4 hours. The efflux media and cells were analyzed to determine the percent of cholesterol release.

### **Reverse cholesterol transport**

Experiments were performed in severe combined immunodeficient (SCID) mice (n = 10). In brief,  $^3\text{H}$ -cholesterol-labeled ixmyelocel-T (n = 3) and J774 cells ( $3-5 \times 10^6$ ) 0.5 ml MEM-HEPES were injected intraperitoneally. Blood was collected at 24 and 48 hours, and plasma was used for scintillation counting. Feces were collected continuously for 48 hours and used for scintillation counting.

### **Real-time PCR**

Both CD14+ ixmyelocel-T and THP-1 macrophages were loaded with 50 µg/mL Ac-LDL for 24 hours in serum-free media containing 0.2% BSA (fatty acid-free). After 24 hours, the cells were washed with PBS and harvested for RNA isolation. For real time PCR, total RNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and 1 µg of RNA was reverse transcribed using the high capacity cDNA reverse transcription kit per the manufacturer's directions (Applied Biosciences, Carlsbad, CA, USA). Relative levels of target gene expression were measured on the 7500 Real-Time PCR system (Applied Biosystems). FAM-based Taqman Gene Expression Assay Mix (Applied Biosystems) specific for each gene of interest and Taqman Universal Master Mix (Applied Biosystems) were used. Relative quantification PCR analysis was performed using the ABI 7500 Software (Applied Biosystems). The relative amount of cDNA was calculated by normalization to GAPDH.

### **Statistical analysis**

Paired *t*-test was performed to compare results. A *p*-value less than 0.05 was considered statistically significant. Data are reported as mean ± SEM.

## **Results**

### **Ixmyelocel-T macrophages influx modified cholesterol**

The ability to ingest modified cholesterol and the degree of Ac-LDL accumulation was examined with fluorescent microscopy and flow cytometry using Dil-Ac-LDL (Figure 1). Fluorescent microscopy revealed that the THP-1 macrophages loaded with Dil-Ac-LDL

appeared to be loaded with large lipid droplets (Figure 1A). Ixmyelocel-T macrophages loading with Dil-Ac-LDL appeared to be loaded with smaller lipid droplets (Figure 1A). Flow cytometry revealed that both ixmyelocel-T and THP-1 ingest similar amounts of Dil-Ac-LDL ( $257 \pm 13$  vs  $246 \pm 10$  –mean fluorescent intensity,  $p = 0.26$  compared with THP-1; Figure 1B). These results provide evidence that ixmyelocel-T macrophages do influx modified cholesterol. These results also suggest that ixmyelocel-T macrophages do not store large droplets, or pools, of cholesterol.

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**Figure 1 Ixmyelocel-T macrophages influx modified cholesterol.** (A) Fluorescent microscopy images and (B) flow cytometry analysis ( $n \geq 5$ ) of ixmyelocel-T and THP-1 macrophages loaded with Dil-Ac-LDL. Quantitative real-time PCR gene expression analysis of scavenger receptors normalized to GAPDH ( $n \geq 5$ ). Expression of (C) CD36 and (D) SCARB1 in THP-1 and ixmyelocel-T macrophages before and after lipid loading. Values are presented as mean  $\pm$  SEM relative to control, \*  $p < 0.01$  vs THP-1 -Ac-LDL, \*\*  $p < 0.001$  vs THP-1 -Ac-LDL. Magnification: 40X. Dil-Ac-LDL, low-density lipoprotein from human plasma, acetylated, Dil complex; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SCARB1, scavenger receptor class B type I; SEM, standard error of the mean; Ac-LDL, acetylated low-density lipoprotein.

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To further understand the mechanisms of Ac-LDL uptake, real-time PCR was used for to examine expression of the modified lipid scavenger receptors CD36 and scavenger receptor (SR)-B1 before and after lipid loading with Ac-LDL. There was not a statistically significant increase in *CD36* expression after lipid loading in either ixmyelocel-T or THP-1 macrophages. THP-1 cells exhibited increased expression of *SR-B1* after lipid loading ( $1.44 \pm 0.11$  vs  $1 \pm 0.05$ -fold change,  $p < 0.01$  compared with THP-1 -Ac-LDL; Figure 1D), whereas there was no change in expression of *SR-B1* in ixmyelocel-T macrophages treated with Ac-LDL ( $0.16 \pm 0.04$  vs  $0.21 \pm 0.05$  fold change;  $p = \text{NS}$  compared with ixmyelocel-T + Ac-LDL; Figure 1D). These data suggest that ixmyelocel-T macrophages influx Ac-LDL through scavenger receptors that ingest modified lipids.

### **Ixmyelocel-T does not become apoptotic after lipid loading**

To determine if lipid loading resulted in apoptosis, both ixmyelocel-T and THP-1 macrophages were loaded with 100  $\mu\text{g}/\text{mL}$  Ac-LDL for 24 hours. Apoptosis was then analyzed using a luminometric caspase 3/7 assay, which examines activation of caspases-3 and -7. As shown in Figure 2, THP-1 macrophages exhibited a statistically significant higher level of Ac-LDL induced apoptosis ( $114 \pm 3.58\%$  vs  $100 \pm 3.58\%$  of control,  $p < 0.01$  compared with THP-1 -Ac-LDL; Figure 2), than ixmyelocel-T ( $87 \pm 10.78\%$  vs  $100 \pm 14.58\%$  of control,  $p = \text{NS}$  compared with ixmyelocel-T -Ac-LDL; Figure 2).

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**Figure 2 Ixmyelocel-T is not sensitive to modified cholesterol-induced lipotoxicity. Percent of apoptosis after cholesterol loading.** Values are presented as mean  $\pm$  SEM relative to control, \*  $p < 0.01$  vs THP-1 -Ac-LDL. SEM, standard error of the mean; Ac-LDL, acetylated low-density lipoprotein.

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## Ixmyelocel-T secretes minimal amounts of pro-inflammatory cytokines after lipid loading

When macrophages are unable to maintain cholesterol homeostasis due to ineffective cholesterol efflux, it generates a pro-inflammatory response [5]. Therefore, IL-1 $\beta$ , TNF $\alpha$ , IL-6, and MCP-1 secretion was measured in lipid-loaded THP-1 and ixmyelocel-T macrophages before and after LPS stimulation (Table 1). When stimulated with LPS, THP-1 macrophages secreted significantly elevated amounts of IL-1 $\beta$ , TNF $\alpha$ , IL-6, and MCP-1 (Table 1). When ixmyelocel-T macrophages were stimulated with LPS, there was a statistically significant increase in IL-6; however, the level of IL-6 is lower compared with those from THP-1 macrophages (Table 1). Overall, THP-1 macrophages secreted significantly higher amounts of the inflammatory cytokines after LPS stimulation compared with ixmyelocel-T macrophages. THP-1 macrophages exhibited a 514-fold increase for IL-1 $\beta$ , a 497-fold increase for TNF $\alpha$ , a 162-fold increase for IL-6, and a 32-fold increase for MCP-1 (Table 1). However, ixmyelocel-T macrophages exhibited a 5-fold increase for IL-1 $\beta$ , a 2-fold increase for TNF $\alpha$ , a 3-fold increase for IL-6, and a 1.5-fold increase for MCP-1 (Table 1). These data demonstrate that ixmyelocel-T macrophages remain anti-inflammatory even after lipid loading.

**Table 1 Ixmyelocel-T secretes minimal amounts of pro-inflammatory cytokines after lipid loading**

	THP-1 (-LPS)	THP-1 (+LPS)	IXT (-LPS)	IXT (+LPS)
<b>IL-1<math>\beta</math></b>	25.7 $\pm$ 6	13213 $\pm$ 269 ***	2.2 $\pm$ 0.8 &&	10 $\pm$ 1.9 ***
<b>TNF<math>\alpha</math></b>	135 $\pm$ 30	67063 $\pm$ 8539 ***	86 $\pm$ 54	151 $\pm$ 80 ***
<b>IL-6</b>	97 $\pm$ 19	15733 $\pm$ 1874 ***	769 $\pm$ 353 &	2376 $\pm$ 551 #, ***
<b>MCP-1</b>	940 $\pm$ 120	29611 $\pm$ 2713 ***	5575 $\pm$ 1912 &	8480 $\pm$ 2491 ***

Cytokines were quantified in supernatants from lipid-loaded ixmyelocel-T and THP-1 macrophages treated with and without LPS ( $n \geq 6$ ). The amount of cytokine expressed was normalized to the cell number of each sample. Values are presented as mean  $\pm$  SEM relative to control, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs THP-1 - LPS; #  $p < 0.05$  vs IXT - LPS, &  $p < 0.05$ , &&  $p < 0.01$  vs THP-1 - LPS. LPS, lipopolysaccharide; SEM, standard error of the mean; IXT, ixmyelocel-T.

## Ixmyelocel-T displays enhanced cholesterol efflux capacity

Using real-time PCR, expression of key genes involved in cholesterol efflux (eg, *ABCA1* and *ABCG1*) was examined with and without cholesterol loading. There was a statistically significant increase in expression of *ABCA1* in ixmyelocel-T macrophages after cholesterol loading (1.49  $\pm$  0.32 vs 0.42  $\pm$  0.11-fold change,  $p < 0.01$  compared with ixmyelocel-T -Ac-LDL; Figure 3A), whereas *ABCA1* was significantly decreased in THP-1 macrophages loaded with cholesterol (0.78  $\pm$  0.05 vs 1  $\pm$  0.07-fold change,  $p < 0.05$  compared with THP-1 -Ac-LDL; Figure 3A). Additionally, ixmyelocel-T macrophages expressed significantly more *ABCA1* after lipid loading compared with THP-1 macrophages (1.49  $\pm$  0.32 vs 0.78  $\pm$  0.05-fold change,  $p < 0.05$  compared with THP-1 + Ac-LDL; Figure 3A). Expression of *ABCG1* was significantly increased in ixmyelocel-T macrophages after cholesterol loading (0.41  $\pm$  0.13 vs 0.07  $\pm$  0.03-fold change,  $p < 0.05$  compared with ixmyelocel-T -Ac-LDL; Figure 3B), whereas *ABCG1* was significantly decreased in THP-1 macrophages loaded with cholesterol (0.75  $\pm$  0.04 vs 1  $\pm$  0.04-fold change,  $p < 0.001$  compared with THP-1 -Ac-LDL; Figure 3B).



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**Figure 3 Ixmyelocel-T displays enhanced cholesterol efflux capacity.** Quantitative real-time PCR gene expression analysis of scavenger receptors normalized to GAPDH ( $n \geq 5$ ). Expression of (A) ABCA1, (B) ABCG1, (C) ACAT1, and (D) CEH in THP-1 and ixmyelocel-T macrophages before and after lipid loading. (E) Cholesterol efflux of both THP-1 macrophages and ixmyelocel-T to the lipid acceptors ApoA-I and HDL3 ( $n \geq 4$ ). Values are presented as mean  $\pm$  SEM relative to control, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs THP-1 -Ac-LDL; # $p < 0.05$ , ## $p < 0.01$  vs IXT -Ac-LDL. PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ABC, ATP-binding cassette; ACAT, acyl-CoA:cholesterol acyl-transferase; CEH, cholesterol ester hydrolase; ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; SEM, standard error of the mean; Ac-LDL, acetylated low-density lipoprotein.

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*ACAT1* and *CEH* were also examined in both cell populations. Ixmyelocel-T macrophages expressed significantly lower *ACAT1* compared with THP-1 macrophages ( $0.56 \pm 0.08$  vs  $1 \pm 0.03$ -fold change,  $p < 0.001$  compared with THP-1 -Ac-LDL; Figure 3C). After lipid loading, there was no change in *ACAT1* expression in ixmyelocel-T macrophages ( $0.63 \pm 0.11$  vs  $0.56 \pm 0.08$ -fold change,  $p = \text{NS}$  compared with ixmyelocel-T -Ac-LDL; Figure 3C), whereas THP-1 macrophages expressed significantly elevated levels of *ACAT1* ( $1.2 \pm 0.03$  vs  $1 \pm 0.03$ -fold change,  $p < 0.001$  compared with THP-1 -Ac-LDL; Figure 3C). After lipid loading, there was a statistically significant increase in *CEH* expression in ixmyelocel-T macrophages ( $1.42 \pm 0.22$  vs  $0.93 \pm 0.15$ -fold change,  $p < 0.01$  compared with ixmyelocel-T -Ac-LDL; Figure 3D), whereas THP-1 macrophages expressed significantly decreased levels of *CEH* ( $0.71 \pm 0.03$  vs  $1 \pm 0.06$ -fold change,  $p < 0.001$  compared with THP-1 -Ac-LDL; Figure 3D). Since these genes, which are involved in cholesterol efflux, were upregulated in ixmyelocel-T, an *in vitro* cholesterol efflux assay was employed to determine the ability of both cells to efflux cholesterol. Apolipoprotein A-I (ApoA-I)-mediated cholesterol efflux was significantly higher in  $^3\text{H}$ -cholesterol-AcLDL-loaded ixmyelocel-T compared with THP-1 macrophages ( $13.91\% \pm 1.90$  vs  $3.42\% \pm 0.31$ ,  $p < 0.001$  compared with THP-1; Figure 3E). HDL<sub>3</sub>-mediated cholesterol efflux was significantly higher in  $^3\text{H}$ -cholesterol-AcLDL-loaded ixmyelocel-T compared to THP-1 macrophages ( $2.58\% \pm 0.052$  vs  $0.85\% \pm 0.09$ ,  $p < 0.01$  compared with THP-1; Figure 3E). Collectively, these data indicate that ixmyelocel-T macrophages exhibit enhanced capacities to efflux cholesterol.

### **Ixmyelocel-T promotes reverse cholesterol transport**

The classic reverse cholesterol transport (RCT) pathway involves cholesterol efflux from macrophage foam cells to cholesterol acceptors and the excretion of cholesterol into the feces [11,31]. The *in vivo* RCT assay was employed to determine if ixmyelocel-T macrophages are capable of effluxing cholesterol *in vivo*. As shown in Figure 4, ixmyelocel-T is capable of promoting RCT *in vivo*. After injection of  $^3\text{H}$ -cholesterol-labeled ixmyelocel-T into the peritoneal cavity of SCID mice, plasma  $^3\text{H}$ -cholesterol levels were similar to the control cell line J774 cells after both 24 hours ( $3.21 \pm 0.23\%$  vs  $3.48 \pm 0.39\%$  injected cpm,  $p = \text{NS}$  compared to J774; Figure 4A) and 48 hours ( $2.92 \pm 0.22\%$  vs  $2.51 \pm 0.22\%$  injected cpm,  $p = \text{NS}$  compared to J774; Figure 4A). There was statistically significant more  $^3\text{H}$ -tracer found in the livers of mice injected with ixmyelocel-T compared to the J774 cells ( $5.49 \pm 0.30\%$  vs  $3.79 \pm 0.39\%$  injected cpm,  $p < 0.05$  compared to J774; Figure 4B), and no difference found in the feces ( $4.16 \pm 0.14\%$  vs  $4.85 \pm 0.57\%$  injected cpm,  $p = \text{NS}$  compared with J774; Figure 4C). These data demonstrate that ixmyelocel-T is able to efflux cholesterol *in vivo*, promoting cholesterol efflux in this model.

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**Figure 4 Ixmyelocel-T promotes reverse cholesterol transport.** *In vivo* cholesterol efflux was examined in SCID mice after intraperitoneal injections of either <sup>3</sup>H-cholesterol-loaded J774 cells or ixmyelocel-T. (A) Plasma <sup>3</sup>H-cholesterol levels after 24 and 48 hours, (B) <sup>3</sup>H-tracer found in the liver, and (C) <sup>3</sup>H-tracer found in the feces after 48 hours (n ≥ 3 per group). Values are presented as mean ± SEM relative to control, \**p* < 0.05 vs J774. SCID, severe combined immunodeficient; SEM, standard error of the mean.

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## Discussion

The current study provides evidence that ixmyelocel-T macrophages influx and efflux modified cholesterol in an atheroprotective manner. The characteristics of ixmyelocel-T macrophages help maintain cellular cholesterol homeostasis, preventing cellular dysfunction and death, and ultimately promoting reverse cholesterol efflux. These data point to a potential new innovative application of ixmyelocel-T in the treatment of atherosclerotic diseases where massive amounts of modified cholesterol are present. Not only are ixmyelocel-T macrophages less susceptible to becoming foam cells, but they also appear to house the biological machinery necessary to promote RCT, which has the potential to prevent progression or induce regression of atherosclerosis.

Uncontrolled accumulation of modified lipids in macrophages is a continuous process that contributes to all stages of atherosclerosis. Expression of scavenger receptors on macrophages is not downregulated by the accumulation of intracellular lipids, resulting in uncontrolled lipid accumulation and eventual irreversible cell damage and death [32]. This study and previously reported data (Ledford KJ, Parrish C, Zeigler F, Bartel RL: Ixmyelocel-T, an expanded multicellular therapy, contains a population of alternatively activated macrophages, Forthcoming) demonstrated that ixmyelocel-T macrophages express relatively low levels of surface receptors involved in the uptake of modified cholesterol. Specifically, previous data demonstrated that ixmyelocel-T macrophages expressed lower levels of scavenger receptors involved in modified cholesterol uptake compared to M2 macrophages. In addition, ixmyelocel-T macrophages did not upregulate expression of these surface receptors in the presence of modified lipids. Fluorescent microscopy and flow cytometry demonstrated that ixmyelocel-T macrophages exhibited accumulation of modified LDL, showing that they do indeed influx modified cholesterol. However fluorescent microscopy suggested that ixmyelocel-T macrophages do not store large pools of cholesterol esters, suggesting that a majority of this cholesterol remains in the free state ready for efflux. These data suggest that the macrophages generated by ixmyelocel-T may employ protective mechanisms to prevent uncontrolled cholesterol loading, which is toxic to macrophages. These results suggest that ixmyelocel-T macrophages employ a protective mechanism against unregulated modified cholesterol uptake by not upregulating scavenger receptor expression in the presence of modified cholesterol, as well as not storing vast amounts as esters. This observation is in alignment with the reduced apoptosis found in ixmyelocel-T macrophages after cholesterol loading and the minimal increase in pro-inflammatory cytokine secretion after lipid loading and inflammatory challenge. The relatively low expression of scavenger receptors after modified cholesterol loading is one potential mechanism by which ixmyelocel-T is protected from cellular apoptosis after high lipid loading.

ACAT-1 promotes accumulation of intracellular CE in macrophages [33]. Studies have demonstrated that ACAT-1 plays an important role in atherosclerosis and is expressed at elevated levels in foam cells located within atherosclerotic lesions [7,33]. In addition, studies

have also demonstrated that reduced ACAT-1 expression has been reported to inhibit foam cell formation in macrophages [33]. The results of this study show that there was a statistically significant decrease in *ACAT-1* expression in ixmyelocel-T macrophages when compared with THP-1 macrophages. Moreover, after loading with Ac-LDL, expression of *ACAT-1* was not increased in ixmyelocel-T macrophages, a feature THP-1 macrophages did not share. Hydrolysis of stored CE is the first and rate-limiting step in cholesterol efflux from macrophage foam cells, and increased expression of CEH leads to increased mobilization of CE, thereby decreasing lipid accumulation [9]. After loading with modified cholesterol, ixmyelocel-T macrophages exhibited increased *CEH* expression, the expression of which was decreased in THP-1 macrophages. These results may explain why the fluorescent microscopy images indicated that ixmyelocel-T macrophages do not store large pools of cholesterol esters. A decrease in *ACAT-1* and increase in *CEH* would suggest that a majority of the ingested cholesterol remains in the free state ready for efflux. The combination of both increased *CEH* and decreased *ACAT-1* expression after cholesterol loading may be one mechanism by which ixmyelocel-T macrophages reduce cholesterol accumulation, thus preventing lipotoxicity.

Macrophages maintain cholesterol homeostasis with a balance between influx and efflux pathways [5]. To appreciate the potential impact of ixmyelocel-T macrophages on cholesterol homeostasis, it is essential to consider all aspects of cholesterol homeostasis, including efflux from cells. This investigation demonstrated that ixmyelocel-T macrophages display enhanced cholesterol efflux capacity. Specifically, these data demonstrated that after loading with Ac-LDL, ixmyelocel-T macrophages upregulated expression of both cholesterol transporter genes, *ABCA1* and *ABCG1*. Both of these transporters are critical in the efflux of cholesterol from macrophages. In addition, results from the cholesterol efflux assay revealed that ixmyelocel-T macrophages were not only able to efflux cholesterol to the acceptor ApoAI and HDL,<sup>3</sup> but also performed this action at an enhanced level. Ixmyelocel-T macrophages were also able to perform this action *in vivo* in the RCT assay. Taken together, these data highlight the enhanced ability of ixmyelocel-T to promote RCT through cholesterol influx and efflux. In addition, the results highlight one mechanism by which ixmyelocel-T macrophages maintain cholesterol homeostasis.

Atherosclerosis is chronic inflammatory disease that leads to a multitude of diseases including coronary artery disease, peripheral artery disease, and stroke [34]. Several therapies have been investigated in atherosclerosis including anti-inflammatory drugs, cytokine, and chemokine antagonists. However at this time no real promising effects on clinical outcomes have been seen on the reduction of atherosclerosis in CAD [34]. Cell therapies have emerged for the treatment of cardiovascular diseases. Several studies have also provided evidence that cell therapies could be a useful treatment in of atherosclerosis, promoting lesion regression and thereby improving vascular health [34-36]. Furthermore, several studies have demonstrated that atherosclerotic lesions contain both M1 and M2 macrophages, providing evidence that M1 macrophages are found in unstable rupture prone region of atherosclerotic plaques [37-39]. Therefore, ixmyelocel-T may potentially beneficial in the treatment of atherosclerotic disease states where they might promote lesion regression or stabilization through immunomodulation, efferocytosis, and modified cholesterol removal. Treatment with ixmyelocel-T has been investigated in bone regeneration, critical limb ischemia, and ischemic DCM. Further analysis of the subpopulations of cells generated in ixmyelocel-T could highlight new and innovative uses of this multicellular therapy. In this case, further analysis of ixmyelocel-T macrophages suggests that treatment with this cellular therapy might be advantageous in disease states involving atherosclerotic lesions which would benefit from

immunomodulation, efferocytosis, and modified cholesterol removal. Future studies will examine the effects of ixmyelocel-T in pre-clinical models to determine if this therapy can affect the atherosclerotic state. For this cell therapy to affect an atheroma, it might need to be present in the lesion itself. Therefore, future studies will examine whether ixmyelocel-T has the ability to infiltrate atherosclerotic lesions or if it needs to be directly injected into areas where it might have the potential to dampen the immune response, remove necrotic debris, and efflux cholesterol.

## Conclusion

The present data provide evidence that ixmyelocel-T has the potential to become an effective adjuvant in the treatment of diseases due to atherosclerosis. Ixmyelocel-T contains a unique M2-like population of macrophages that are efficient at efferocytosis and maintaining cholesterol homeostasis. The data presented here suggest that ixmyelocel-T macrophages may exert beneficial effects in atherosclerotic disease since they influx cholesterol, remain anti-inflammatory in the face of lipid loading and inflammatory challenge, and display enhanced cholesterol efflux capabilities. The multifactorial characteristics of the ixmyelocel-T M2-like macrophages described in this analysis, as well as the companion manuscript presented in *Stem Cell Research and Therapy*, highlight the potential effectiveness of this expanded autologous multicellular therapy in the treatment of atherosclerotic diseases.

## Abbreviations

ACAT, acyl-CoA:cholesterol acyl-transferase; Ac-LDL, Acetylated low-density lipoprotein; apoA-I, apolipoprotein A-I; BSA, Bovine serum albumin; cDNA, Complementary DNA; CEH, Cholesterol ester hydrolase; CES, Carboxylesterase; DAPI, 4'-6-diamidino-2-phenylindole; Dil-Ac-LDL, Low-density lipoprotein from human plasma acetylated, DiI complex; FAM, 6-carboxyfluorescein; FBS, Fetal bovine serum; FC, Free or unesterified cholesterol; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HDL, High-density lipoprotein; HEPES, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid; IL, Interleukin; LDL, Low-density lipoprotein; LPS, Lipopolysaccharide; MCP-1, Monocyte chemoattractant protein-1; MEM, Minimum essential medium; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; RCT, Reverse cholesterol transport; RNA, Ribonucleic acid; RPMI, Roswell park memorial institute medium; SCARB1, Scavenger receptor class B type I; SCID, Severe combined immunodeficient; SEM, Standard error of the mean; SR, Scavenger receptor; THP-1, Human leukemia monocyte; TNF $\alpha$ , Tumor necrosis factor-alpha

## Competing interests

All authors are employees of Aastrom Biosciences, Inc..

## Authors' contributions

KL conceived and designed research, acquired data, analyzed and interpreted data and results, performed statistical analysis, and drafted the manuscript. NM prepared samples, analyzed data, and reviewed and provided feedback during the development of the manuscript. FZ provided conceptual advice, analyzed data, and participated in discussion of

results. RB contributed to the scientific direction, experimental approach, and interpretation of results. All authors read and approved the final manuscript.

## Acknowledgements

The authors would like to thank Alden Wong, Judith Schmitt, and Hillary Evens for excellent technical assistance. Scientific editorial support was sponsored by Aastrom Biosciences, Inc., and provided by David E. Kaminsky, PhD, of AlphaBioCom, LLC.

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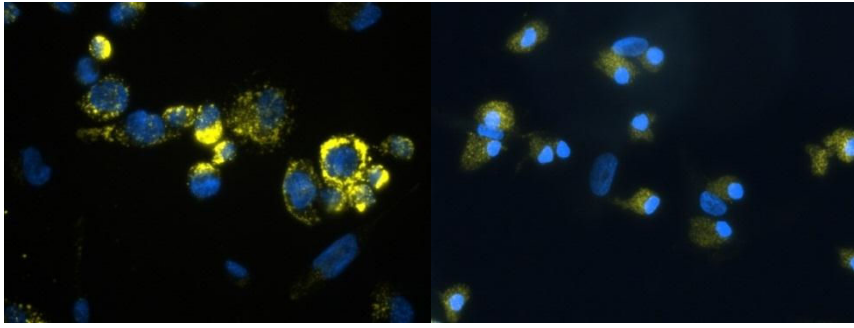
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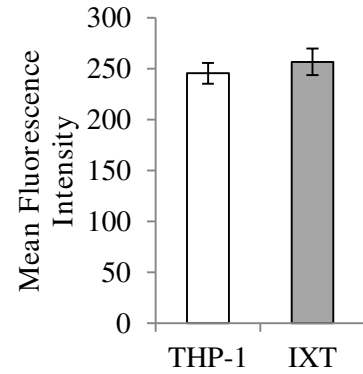
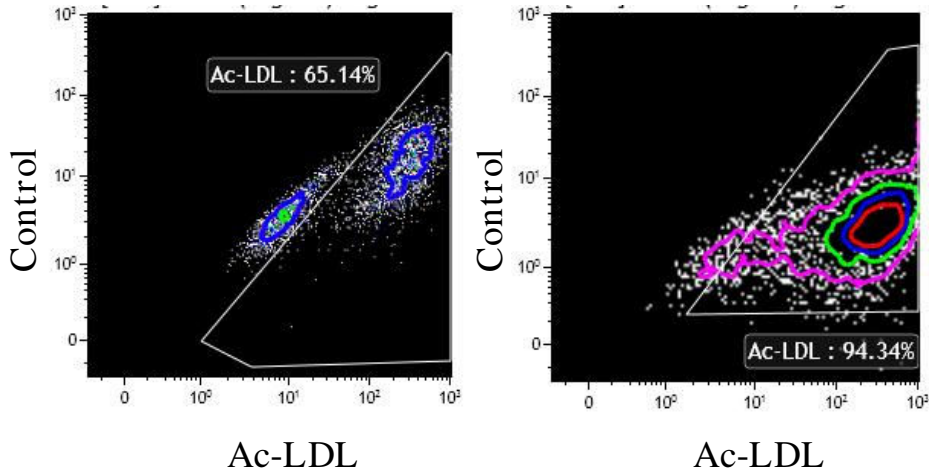
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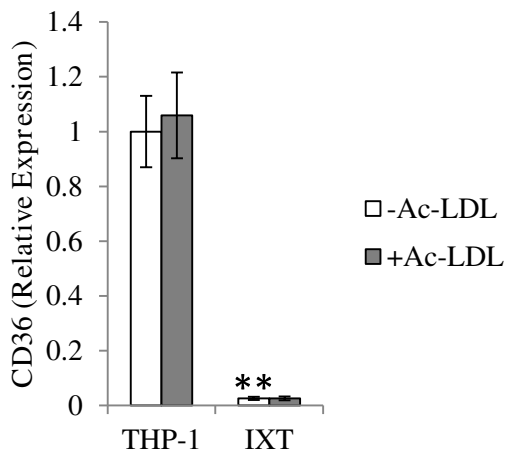
A. THP-1 IXT



B.



C.



D.

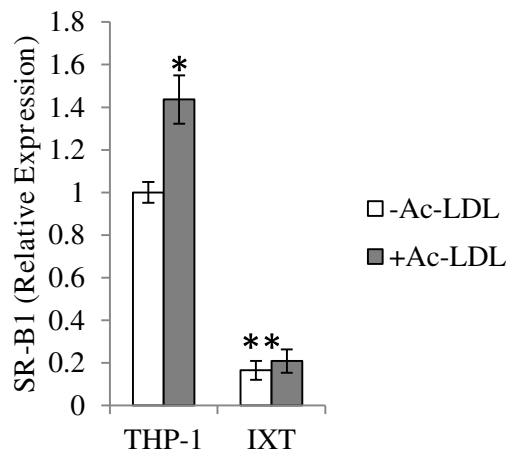


Figure 1

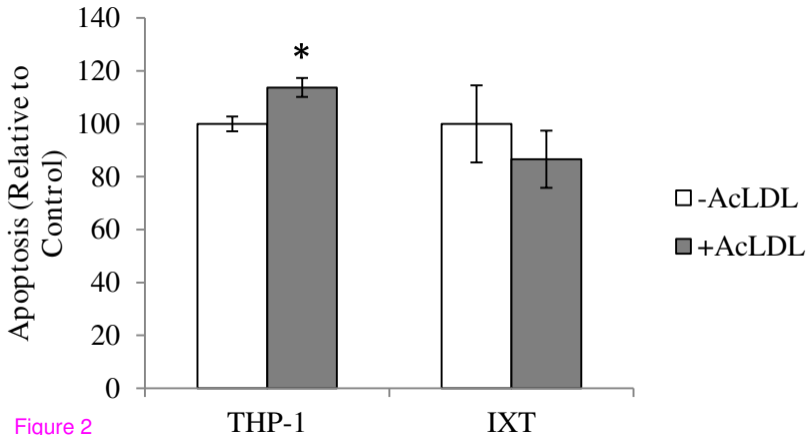


Figure 2

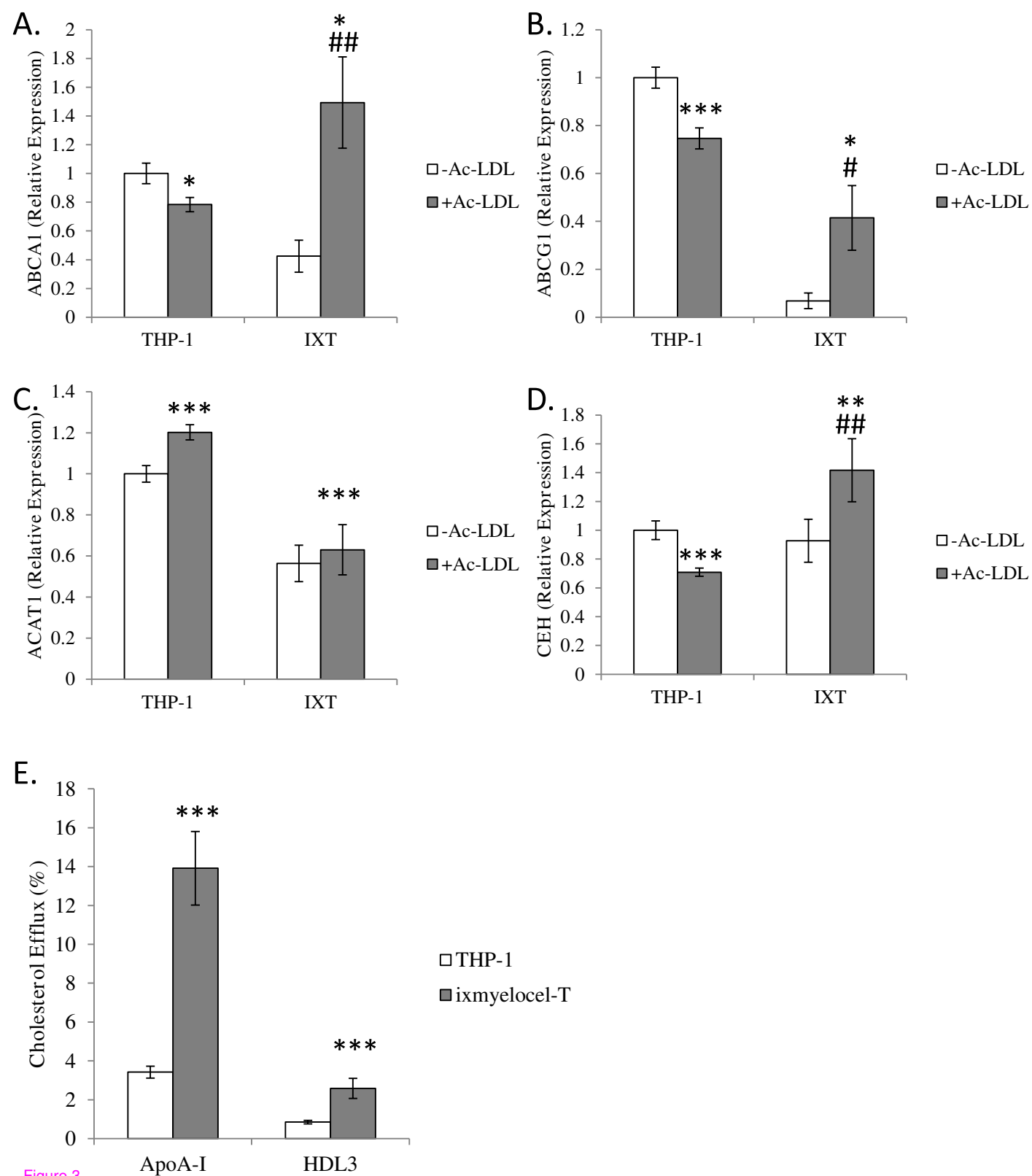


Figure 3

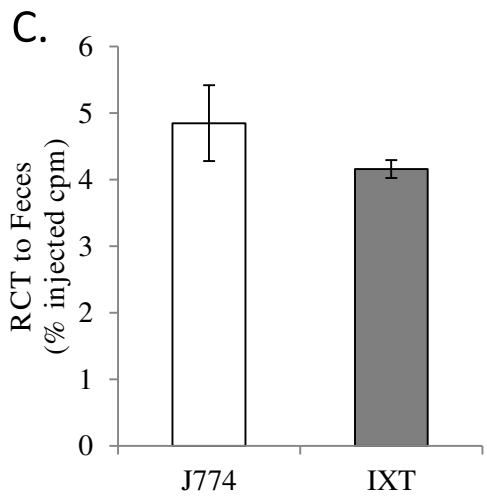
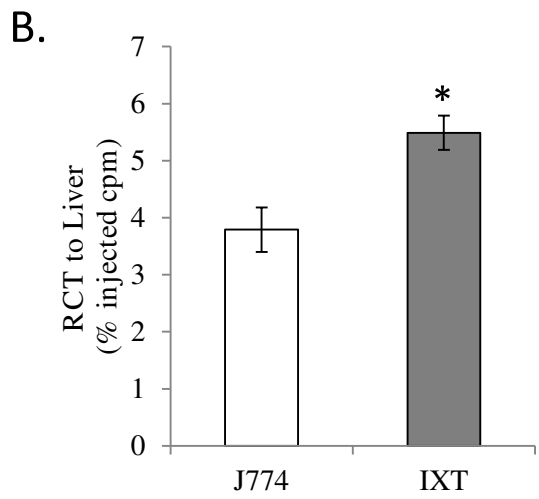
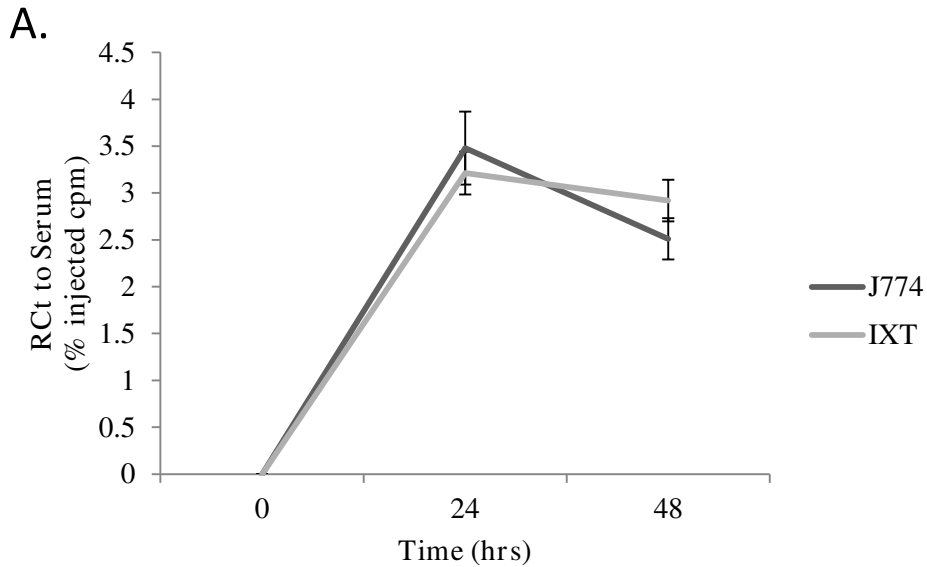


Figure 4