AWARD NUMBER: W81XWH-16-1-0255

TITLE: Regressing Atherosclerosis by Resolving Plaque Inflammation

PRINCIPAL INVESTIGATOR: Dr. Edward A. Fisher

CONTRACTING ORGANIZATION: New York University School of Medicine

New York, NY 10016-6402

REPORT DATE: JULY 2019

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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#### REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JULY 2019	Annual	1 July 2018 – 30 June 2019
4. TITLE AND SUBTITLE		5a. CONTRACT NUMBER
		W81XWH-16-1-0255
Regressing Atherosclerosis by Res	olving Plague Inflammation	5b. GRANT NUMBER
,		PR151468
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER	
Dr. Edward A. Fisher		
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
E-Mail: Edward.Fisher@nyulangon	e.org	
7. PERFORMING ORGANIZATION NAME(	8. PERFORMING ORGANIZATION REPORT NUMBER	
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Fort Detrick, Maryland 21702-5012	NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STAT	EMENT	

Approved for Public Release; Distribution Unlimited

#### 13. SUPPLEMENTARY NOTES

#### 14. ABSTRACT

Macrophages play key roles in progression of atherosclerosis. Our goal is to understand the mechanisms by which atherosclerosis can be clinically regressed by altering the macrophage state in the plaques to resolve the inflammation, as well as to develop new therapeutic strategies to promote atherosclerosis regression by altering the macrophage activation state. We have found that successful atherosclerosis regression requires the alteration of macrophages in the plaques to a tissue repair "alternatively" activated state. This switch in activation state requires the action of TH2 cytokines interleukin (IL)-4 or IL-13. To accomplish our goals, we are testing if these molecules, or derivative of these molecules, will be able to accelerate atherosclerosis regression in mouse models. Additionally, we will develop nanomedicines that can favorably and rapidly affect the content and inflammatory state of macrophages in atherosclerotic plaques to promote regression. Concurrently, we will characterize the macrophages to understand the mechanisms that promote atherosclerosis regression.

#### 15. SUBJECT TERMS NOTHING LISTED

16. SECURITY CLASSIFICATION OF:		17. LIMITATION	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON	
		OF ABSTRACT	OF PAGES	USAMRMC	
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area
Unclassified	Unclassified	Unclassified	Unclassified	42	code)

#### 1. INTRODUCTION

We currently have a limited capacity to **reverse** the high level of atherosclerotic plagues already present in the population. Our vision is to harness the immune system to reverse atherosclerosis. Inadequate resolution of inflammation is fundamental to all stages of atherosclerosis, with macrophages playing key roles in progression of the disease. The goal of our proposal is to understand the mechanisms by which atherosclerosis can be clinically regressed by altering the macrophage state in the plaques to resolve the inflammation, as well as to develop new therapeutic strategies to promote atherosclerosis regression by altering the macrophage activation state. By understanding and harnessing these mechanisms in mouse models of atherosclerosis, the final goal would be to develop new immunotherapeutic approaches that can complement existing lipid lowering treatments, thereby providing benefits to veterans, who experience a high rate of cardiovascular disease. We have recently found that successful atherosclerosis regression requires the alteration of macrophages in the plaques from an inflammatory "classically" activated state to a tissue repair "alternatively" activated state. This switch in activation state requires the action of T<sub>H</sub>2 cytokines interleukin (IL)-4 or IL-13. To accomplish our goals, we are testing if these molecules, or derivative of these molecules, will be able to accelerate atherosclerosis regression in mouse models. Additionally, we will develop nanomedicines that can favorably and rapidly affect the content and inflammatory state of macrophages in atherosclerotic plagues to promote regression. Concurrently, we will characterize the macrophages to understand the mechanisms that promote atherosclerosis regression.

**2. KEYWORDS:** Atherosclerosis, cardiovascular disease, macrophages, interleukin 4, nanoparticles.

#### 3. ACCOMPLISHMENTS:

The major goals and objectives of the project are:

Specific Aim 1: To determine the mechanism(s) regulating inflammation resolution in regressing plaques and to promote resolution by administration of  $T_{\rm H2}$  cytokines (IL-4 and IL-13).

To accomplish this aim, the Major Tasks are:

- (1) Determine the requirement for IL-4 in mediating M2 activation and regression and to generate conditional STAT6 deficient animals to independently determine the requirement for the IL-4 signaling pathway in specific cell types, including macrophages.
- (2) Determine the cellular source of IL-4 in mediating M2 activation and regression.
- (3) Determine the optimal dosage of IL-4 or IL-13 required in vivo for M2 activation of peritoneal macrophages without adverse effects.

- (4) Determine the optimal dosage and efficacy of IL-4 or IL-13 required in vivo to promote plaque regression.
- $(5) Generate ATAC-seq data from M{\small 2}\,macrophages from a the roscleros is plaques.$
- (6) Generate RNA-seq data from M2 macrophages from atherosclerosis plaques.
- (7) Integrate ATAC-seq and RNA-seq data to build a transcriptional network for M2 activation in regressing plaques.

## Specific Aim 2: To develop nanomedicines to favorably and rapidly affect the content and inflammatory state of macrophages in atherosclerotic plaques.

To accomplish this aim, the Major Tasks are:

- (1) Determine the efficacy of nanoparticles containing LXR agonist to promote plaque regression.
- (2) Characterize changes to plaque macrophages after treatment with LXR-agonist-NP.
- (3) Determine the efficacy of nanoparticles containing Netrin1- and Unc5b-siRNA to promote plaque regression.
- (4) Characterize changes to plaque macrophages after treatment with Netrin1-and Unc5b-siRNA –NP.

What was accomplished under these goals? Majoractivities for this reporting period: Aim 1 Major Tasks

**Major Task 1** Determine the requirement and source of IL-4 required for atherosclerosis regression.

## Major Task 1a: Fisher lab: Determine the requirement for IL-4 for atherosclerosis regression.

- In last year's report, we presented preliminary data from an aortic transplant study that atherosclerosis regression was still achieved in the absence of IL4/IL13 in monocytes that are newly recruited to the plaque after lipid lowering. We completed the analyses and that conclusion held up. This implied that neither cytokine was needed to participate in the polarization of plaque macrophages to the inflammation resolving M2 state, a process we showed (1) to be required for atherosclerosis regression. Alternatively, they were required, but that the important pool of cytokines was already present in the plaque before lipid reduction. To test this, we used the IL4/IL13 double knockout (DKO) mice to be the aortic transplant donors. Thus, these mice were first made hypercholesterolemic by an injection of an adenoviral vector expressing PCSK9. After 20 weeks of feeding the atherogenic "western diet, regression was initiated by injections of an anti-sense oligonucleotide (ASO) to apoB to reduce hepatic lipoprotein secretion (which reverses the hypercholesterolemia), and 3 weeks days after that, the aortic samples were collected and analysed. As shown in Figure 1, regression (as assessed as we usually do by plaque macrophage content) was now impaired, indicating that these cytokines were indeed required, but that the plaque content prior to the regression stimulus was apparently sufficient for the polarization stimulus. These results agree with our measurements that showed that neither cytokine changed significantly in plaques before and after regression and with data using mice with a reporter for IL4 (the "4get mouse") that showed the presence in plagues of IL4 expressing cells, but there was no change in their number associated with regression.
- · How do we explain, then, that unchanging concentrations of these cytokines apparently

induced macrophages to become inflammation resolving? Upon examination of our prior transcriptomic data from macrophages selected from progressing and regressing plaques (2), we noticed an increase in Wnt signaling during regression. In recent experiments, we have noted in vitro that the addition of a classical Wnt ligand (Wnt 3a) augments the response of macrophages to IL4 or IL13 (A. Weinstock, data not shown). Our working hypothesis is that there is induction of Wnt signaling in plaque macrophages when regression is initiated by lipid lowering, which amplifies their response to IL4/IL13.

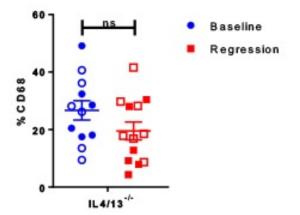


Fig. 1 Deficiency of IL4 and IL13 before reversal of hyperlipidemia impairs atherosclerosis regression. IL4/IL13 DKO Mice were treated as described in text. At the end of the baseline (blue) and the lipid reduction (red)periods, the aortic root was analyzed for the content of macrophages (CD68+ cells). Note the non-significant result between the baseline and "regression" groups.

**Major Task 1b: Fisher and Loke labs:** Generate conditional STAT6 deficient animals to independently determine the requirement for the IL-4 signaling pathway in specific cell types, including macrophages.

• We received conditional STAT6 deficient animals from Ingenious Targeting Laboratories. After rederivation into our animal facility we have crossed these animals to macrophage specific Cre expressing mice. These include the CD169-Cre to delete STAT6 in tissue resident macrophages and CX3CR1-Cre and CSF1R-Cre mice to delete them in all other macrophages. In the next funding period, there should be sufficient mice available for experiments to be performed.

## Major Task 2: Fisher Lab: Determine the cellular source of IL-4 in mediating M2 activation and regression.

• As noted in the update to Major Task 1A, we are more focused on how the cells in the plaque become responsive to whatever IL4/IL13 is there already at the time we initiate regression. Once we make progress on that front, we will return to the source. Preliminary evidence from the 4GET studies is that eosinophils may be the cellular source, but this will need to be confirmed. If so, a promising set of experiments would be to delete eosinophils using a mouse model called "double GATA" and see if this impairs regression.

**MajorTask3: Lokelab:** Determine the optimal dosage of IL-4 or IL-13 required in vivo for M2 activation of peritoneal macrophages without adverse effects.

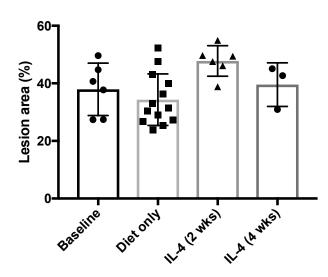
• In the previous funding period, we had optimized the dosage of IL-4 and IL-13 for the treatment of plaques.

**Major Task 4: Loke lab:** Determine the optimal dosage and efficacy of IL-4 or IL-13 required *in vivo* to promote plaque regression.

- We discovered and published that monocyte derived macrophages are the main source of M2 macrophages required for atherosclerosis regression {Rahman, 2017 #1337} and developed a better mouse model for characterization of these macrophages during atherosclerosis. We use a "fate-mapping" approach to track monocyte derived macrophages {Gundra, 2017 #1299}, which can be combined with a (PCSK9)-encoding adeno-associated viral vector system {Peled, 2017 #1338}, for better precision without genetic crosses onto the LDLR-/- background. Utilizing this system, we can isolate tdTomato positive macrophages for downstream analysis.
- We have performed two independent experiments utilizing these tdTomato – PCSK9 mice to develop and then regress atherosclerosis. In the first experiment, we utilized a **2 week** atherosclerosis regression strategy. In the second experiment, we utilized a **4 week** atherosclerosis regression strategy. The protocol was adapted for the second experiment because the IL-4 treatment for the 2 week experiment did not perform as expected (see below).
- For the first experiment (2 week treatment), we treated 17 Cx3cr1-Cre Rosa flox-tdTomato mice (9 females and 8 males) with an AAV

expressing a hyper-active form of the PCSK9 gene and fed the mice on a Western Diet (WD). Serum cholesterol levels were monitored at 2 weeks, 4 weeks, and 17 weeks post AAV injection. After 17 weeks post AAV injection, 14 mice (7 males and 7 females) had serum cholesterol levels over 700mg/dL. At 18 weeks post AAV injection, the baseline group (4 mice, 3 females/1 male) was sacrificed; both aortic roots and arches were harvested as well as serum cholesterol to determine baseline levels of plague and cholesterol. The remaining mice were separated into two groups, a control group switched to chow alone (4) mice, 2 females/2 males) and a treatment group switched to chow and treated with IL-4 (6 mice, 2 females/4 males). The treatment group was given 4 treatments of IL4-Fc (10 g per dose) for two weeks. After 2 weeks treatment and on the chow diet, which is 20 weeks post AAV injection, mice in the control group and the treatment group were sacrificed; both aortic roots and arches were harvested as well as serum cholesterol. After sectioning the aortic roots, and staining for CD68 plaque area, we observed a trend towards increase in plaque area in the IL-4 treatment group (Figure 3), which was the opposite of what we expected. One possibility is that IL-4 treatment expanded the population of M2 macrophages in the plaques, but have yet to begin the process of plaque resolution.

Hence, to address the question of whether plagues need more time to remodel after IL-4 treatment, we performed a second experiment. In the second experiment, 15 Cx3cr1-Cre Rosa flox-tdTomato mice (5 females/10 males) were treated with an AAV expressing a hyper-active form of the PCSK9 gene and fed the mice on a Western Diet (WD). Serum cholesterol levels were monitored at 2 weeks, 4 weeks, 16 weeks and 19 weeks post AAV injection. At 19 weeks post AAV injection, 12 mice (5 females/7 males) were found to have serum cholesterol levels over 700mg/dL. At 20 weeks post AAV injection, mice were separated into two groups, a control group (6 mice, 3 females/3 males) and a IL-4 treatment group (6 mice, 2 females/4 males) for four weeks. The treatment group was switched to normal chow diet and given 4 treatments of IL4-Fc (10mg per dose) for the first two weeks, and then both groups stayed on the normal chow diet to enable time for plaque resolution. However, IL-4 treatment unexpectedly increased plague size in the treated mice. Hence, instead of promoting plaque regression, it promoted plague regression. We have now completed the repeat experiments with more animals and confirmed these findings (Figure 2). We noticed that these animals had hugely expanded spleens. It is likely that IL-4 treatment increased proliferation (3), as well as the splenic reservoir of monocytes (4). Since IL-4 had an adverse effect on plagues, we have not proceeded with IL-13 treatment.



## Fig. 2 IL4 treatment does not promote atherosclerosis regression.

Atherosclerotic mice were treated with IL-4 as described in the text for 2wks and 4 wks and compared to mice at the baseline period, as well as mice that only underwent diet switch without treatment. The aortic root was analyzed for lesion area and there was no significant differences between any of the groups.

**Major Task 5: Loke lab:** Generate ATAC-seq data from monocyte derived M2 macrophages from atherosclerosis plaques.

 We have not been able to obtain sequencing libraries of sufficient quality from sorted cells. Our success in obtaining single-cell RNA seq results (see below) has shifted our focus towards the interpretation of those results.

## Major Task 6: Fisher and Loke labs: Generate RNA-seq data from monocyte derived M2 macrophages from atherosclerosis plaques.

• Due to the recent availability of the 10X genomics platform for single cell RNA seq analysis, we have applied this approach towards characterizing the transcriptional profiles of monocyte derived macrophages in atherosclerosis progression and regression. We used a combination of single-cell RNA sequencing and genetic fate mapping approaches described above to profile 3157 and 2198 aortic cells derived from CX3CR1+ precursor in atherosclerotic mice during plaque progression and regression. These results have just been successfully published.

Major Task 7: Integrate ATAC-seq and RNA-seq data to build a transcriptional network for M2 activation in regressing plaques.

We have not been able to obtain ATAC-seq data of sufficient quality, but are currently developing computational models to build a transcriptional network based on single-cell RNAseq results that we have already obtained from macrophages in progressing and regressing plaques (see above and in attached publication).

#### Aim 2 Major Tasks:

Major Task 1: Fisher lab: Determine the efficacy of nanoparticles containing LXR agonist to promote plaque regression in LDLR-/- mice.

• All of these studies were successfully completed in the first funding period.

Major Task 2: Fisher lab: Characterize changes to plaque macrophages after treatment with LXR-agonist-NP.

• All of these studies were successfully completed in the first funding period and has now been successfully published (see below).

## Major Task 3: Moore lab: Determine the efficacy of nanoparticles containing Netrin1- and Unc5b-siRNA to promote plaque regression in LDLR-/- mice.

• As mentioned last year, the "empty" nanoparticles (control) were also anti-atherogenic, most likely because they effluxed cholesterol from plaque macrophages. After reviewing all of the data, we decided to terminate this study.

**Major Task 4:** Moore lab: Characterize changes to plaque macrophages after treatment with Netrin1- and Unc5b-siRNA -NP.

• As noted just above, the study was terminated, so this task was not undertaken.

## What opportunities for training and professional development did the project provide?

#### **Postdoctoral Fellows:**

- **Dr. Jian-Da Lin, Ph.D.,** is an expert in immunology who has been working on this project and as a result is being trained in the field of atherosclerosis. His previous research experience was on inflammatory responses to viral infections in the gut, and he is applying this immunology expertise to the project while learning techniques specific for atherosclerosis.
- **Dr. Ada Weinstock**, **Ph.D.**, is also well trained in immunology, who through this grant, has been able to gain experience in atherosclerosis models. She has been able to use this experience to the IL4/IL13 component of Task 1a.
- **Milessa Afonso**, **Ph.D.**, is an expert in lipid metabolism, who through this grant, has gained experience in mouse models of atherosclerosis and its analysis. Her work on this grant ended during this past funding period (Tasks 3 and 4).

**Graduate Students:** None trained under this grant.

#### 5. CHANGES/PROBLEMS:

We continue to have problems in the generation of quality ATAC-seq sequencing libraries from sufficient numbers of cells obtained from the aorta. We have focused on using new technology for single cell RNA seq instead.

#### 6. PRODUCTS:

Lin JD, Nishi H, Poles J, Niu X, Mccauley C, Rahman K, Brown EJ, Yeung ST, Vozhilla N, Weinstock A, Ramsey SA, Fisher EA, Loke P. Single-cell analysis of fate-mapped macrophages reveals heterogeneity, including stem-like properties, during atherosclerosis progression and regression. *JCI Insight*. 2019 Feb 21;4(4). pii: 124574. doi: 10.1172/jci.insight.124574. eCollection 2019 Feb 21.

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Harris NL, Loke P. Recent Advances in Type-2-Cell-Mediated Immunity: Insights from Helminth Infection. *Immunity*. 2017 Dec 19;47(6):1024-1036.

#### 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

## Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes, Dr. Fisher has changes in other support.

#### **Recently Completed**

Title:	Regulation of LXR Alpha by Glucose and Cholesterol in Diabetes and
	Atherosclerosis
Time Commitment:	0.6 Cal Months
Supporting Agency:	NHLBI
Grants Officer:	Norma DeGuzman
Performance Period:	12/23/2013-11/30/2018
Level of Funding:	\$150,000 for Dr. Fisher (MPI grant with Dr. Garabedian)
Project Goals:	This project supports studies on the effects of post-translational modifications of
	LXR alpha on the regulation of gene expression occurring in macrophages under
	conditions of hyperlipidemia and hyperglycemia.
Specific Aims:	Aim 1) To investigate the regulation and modification of LXRα under conditions of
	atherosclerosis progression and regression in normoglycemic and diabetic mice.
	Aim 2) To determine the functional consequences of LXRα S198 phosphorylation
	in atherosclerosis.
	Aim 3) To investigate the effect of LXR phosphorylation on the LXRα
	transcriptome and cistrome in macrophages.
Overlap:	None

#### What other organizations were involved as partners?

Nothing to Report

#### 8. SPECIAL REPORTING REQUIREMENTS:

**COLLABORATIVE AWARDS:** We have included a duplicative report for both the Initiating PI and the Collaborating/Partnering PI and noted the lab assignments to the different labs for the Major Tasks.

#### 9 APPENDICES: see attached documents

#### **References:**

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## **JCI** insight

## Single-cell analysis of fate-mapped macrophages reveals heterogeneity, including stem-like properties, during atherosclerosis progression and regression

Jian-Da Lin, ..., Edward A. Fisher, P'ng Loke

JCI Insight. 2019;4(4):e124574. https://doi.org/10.1172/jci.insight.124574.

Research Article Cardiology

Immunology

Atherosclerosis is a leading cause of death worldwide in industrialized countries. Disease progression and regression are associated with different activation states of macrophages derived from inflammatory monocytes entering the plagues. The features of monocyte-tomacrophage transition and the full spectrum of macrophage activation states during either plaque progression or regression, however, are incompletely established. Here, we use a combination of single-cell RNA sequencing and genetic fate mapping to profile, for the first time to our knowledge, plaque cells derived from CX3CR1+ precursors in mice during both progression and regression of atherosclerosis. The analyses revealed a spectrum of macrophage activation states with greater complexity than the traditional M1 and M2 polarization states, with progression associated with differentiation of CXC3R1<sup>+</sup> monocytes into more distinct states than during regression. We also identified an unexpected cluster of proliferating monocytes with a stem cell-like signature, suggesting that monocytes may persist in a proliferating self-renewal state in inflamed tissue, rather than differentiating immediately into macrophages after entering the tissue.

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# Single-cell analysis of fate-mapped macrophages reveals heterogeneity, including stem-like properties, during atherosclerosis progression and regression

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Atherosclerosis is a leading cause of death worldwide in industrialized countries. Disease progression and regression are associated with different activation states of macrophages derived from inflammatory monocytes entering the plaques. The features of monocyte-to-macrophage transition and the full spectrum of macrophage activation states during either plaque progression or regression, however, are incompletely established. Here, we use a combination of single-cell RNA sequencing and genetic fate mapping to profile, for the first time to our knowledge, plaque cells derived from CX3CR1\* precursors in mice during both progression and regression of atherosclerosis. The analyses revealed a spectrum of macrophage activation states with greater complexity than the traditional M1 and M2 polarization states, with progression associated with differentiation of CXC3R1\* monocytes into more distinct states than during regression. We also identified an unexpected cluster of proliferating monocytes with a stem cell-like signature, suggesting that monocytes may persist in a proliferating self-renewal state in inflamed tissue, rather than differentiating immediately into macrophages after entering the tissue.

#### Introduction

Atherosclerosis underlies coronary artery disease, a leading cause of death in the world. Atherosclerosis already begins its progression in childhood (1), making plaque reversal an important clinical goal during adulthood. There is increasing recognition that plaque progression represents a chronic condition that results from a failure to resolve inflammation (2). The central inflammatory cell in the plaque is the macrophage (3). Though macrophages in tissues can originate from resident macrophages that are seeded in the tissues during embryonic development (e.g., refs. 4, 5), the bulk of plaque macrophages is most likely derived from blood monocytes recruited during disease progression (3). Macrophage proliferation has also been identified as a feature of plaques (6), although the origin of these proliferative cells is unclear.

The two subsets of blood monocytes in mice are often defined by the expression of chemokine receptors: Ccr2+Cx3cr1+(Ly6Chi) for classical monocytes and Ccr2-Cx3cr1+(Ly6Cho) for patrolling nonclassical monocytes, and they have distinct migratory and inflammatory properties (7). Ly6Chi classical monocytes utilize CCR2 and CX3CR1 to enter atherosclerotic lesions in *Apoe*-/- mice (8, 9) and are thought to become classically activated, or M1, macrophages under most inflammatory conditions (9–11). However, alternatively activated M2 macrophages can also be derived from Ly6Chi CCR2-dependent monocytes during helminth infection (12), in allergic inflammation (13), and, as noted below, in regressing atherosclerotic plaques (14). Hence, as newly emigrating Ly6Chi monocytes are exposed to different environmental stimuli in the tissues, they will respond to the signals that result in different activation states.

Based on histochemical markers, the majority of macrophages in both mouse and human progressing plaques resemble the activated classical M1 phenotypic state. We have established a number of different

**Authorship note:** EAF and PL contributed equally to this work.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Submitted:** August 29, 2018 **Accepted:** January 17, 2019 **Published:** February 21, 2019

#### Reference information:

JCI Insight. 2019;4(4):e124574. https://doi.org/10.1172/jci. insight.124574.



mouse models to find that plaque regression is characterized not only by reduced classically activated M1 macrophages, but also by the enrichment of cells expressing markers of alternatively activated (M2 or M[IL-4]) macrophages (3, 15, 16). Alternatively activated M2 macrophages have been shown to participate in resolving inflammation and repairing tissue damage, consistent with features of plaque regression.

This type of macrophage can be derived from tissue-resident macrophages or macrophages derived from classical (Ly6Chi) or nonclassical patrolling (Ly6Cho) monocytes. We recently demonstrated that plaque regression is driven by the CCR2-dependent recruitment of macrophages derived from inflammatory Ly6Chi monocytes that adopt features of the M2 state in a STAT6-dependent manner (14). This suggests that in both progressing and regressing plaques, classically and alternatively activated macrophages are both derived from inflammatory Ly6Chi monocytes. The full scope of different macrophage activation states after transition from monocytes, however, is only just being revealed by single-cell analysis during plaque progression (17, 18) and, notably, is still unknown for plaque regression. Also, the traditional definition of M1 and M2 macrophage activation states often represents polar extremes that are based on in vitro activation conditions with high concentrations of stimuli and on a small number of markers. Thus, the typical conditions of studies in vitro probably do not reflect the more complex in vivo physiological state in a number of key ways, further contributing to the incomplete understanding of monocyte-to-macrophage maturation process in inflammatory conditions, with the process likely to be tissue specific (19).

To improve the understanding of the origins and fates of macrophages in atherosclerotic plaques undergoing dynamic changes, we have combined single-cell RNA-Seq with genetic fate mapping of myeloid cells derived from CX3CR1<sup>+</sup> precursors for application in a mouse model in which plaques form and then are induced to regress. This not only greatly increases the resolution of detail over what is afforded by the limited number of markers typically used to study macrophage phenotypes, but also allows extensive characterizations in the in vivo setting. As we will describe, in atherosclerotic plaques there is a spectrum of macrophage activation states with greater complexity than the traditional M1/M2 definitions, with progressing plaques containing more discernible macrophage activation states than during regression. We also found a population of proliferating cells, remarkably, with monocyte markers and stem cell–like signatures, that may represent a new self-renewing source of macrophages in both progressing and regressing plaques.

#### Results

Fate mapping the conversions of plaque macrophages derived from CX3CR1<sup>+</sup> precursors during atherosclerosis progression and regression. All blood monocytes that migrate into atherosclerotic plaques express CX3CR1 (20, 21); hence, we first examined the fate of these monocytes during atherosclerosis progression by generating BM chimeras of Ldlr<sup>-/-</sup> mice reconstituted with BM from Cx3cr1<sup>CreERT2-IRES-YFF/+</sup>Rosa26<sup>fl-tdTomato/+</sup> mice, which were then fed an atherogenic Western diet (WD). We took this approach because we previously utilized this tamoxifen-inducible (TAM-inducible) Cre recombinase (CreER) system under the control of the Cx3cr1 promoter to fate map monocyte-derived macrophages without adoptive transfer in a schistosomiasis model (5). TAM treatment irreversibly and genetically labels CX3CR1<sup>+</sup> cells and causes them to express tdTomato. Thus, the Ldlr<sup>-/-</sup>:Cx3cr1<sup>CreERT2-IRES-YFF/+</sup>Rosa26<sup>fl-tdTomato/+</sup> BM chimeras were treated with 2 doses of TAM at 14 and 15 weeks of WD, and the aortic root plaques were examined after 18 total weeks of WD feeding, which resulted in advanced plaques (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.124574DS1).

As shown in Figure 1A, newly recruited CX3CR1-EYFP<sup>+</sup> but TdTomato<sup>-</sup> cells were mostly observed in an abluminal, subendothelial location. In contrast, TdTomato<sup>+</sup>EYFP<sup>+</sup> cells were observed further inward, toward the lipid core. Both populations were found in the adventitia, with somewhat more being TdTomato<sup>+</sup>EYFP<sup>+</sup> (Figure 1A). Additionally, we observed considerable heterogeneity in shape (including elongated cells, small and foamy macrophages) among the TdTomato<sup>+</sup> cells derived from CX3CR1<sup>+</sup> precursors (Supplemental Figure 1B).

To analyze the phenotype cells derived from CX3CR1<sup>+</sup> precursors during atherosclerosis progression and regression without generating BM chimeras in *Ldlr*<sup>-/-</sup> mice, we utilized a variant of our recently reported model of atherosclerosis regression. Plaque progression is initiated by injecting into *Cx3cr1*<sup>CreERT2-IRES-YFF/+</sup>Rosa26<sup>(I-tdTomato/+</sup> mice an adeno-associated viral vector expressing a gain-of-function mutant of protein convertase subtilisin/kexin type 9 (AAVmPCSK9), which results in LDL receptor deficiency and hypercholesterolemia. After plaques form, to initiate regression, plasma lipid levels are lowered by using an antisense oligonucleotide (ASO) to apolipoprotein B (ApoB) (22, 23), which



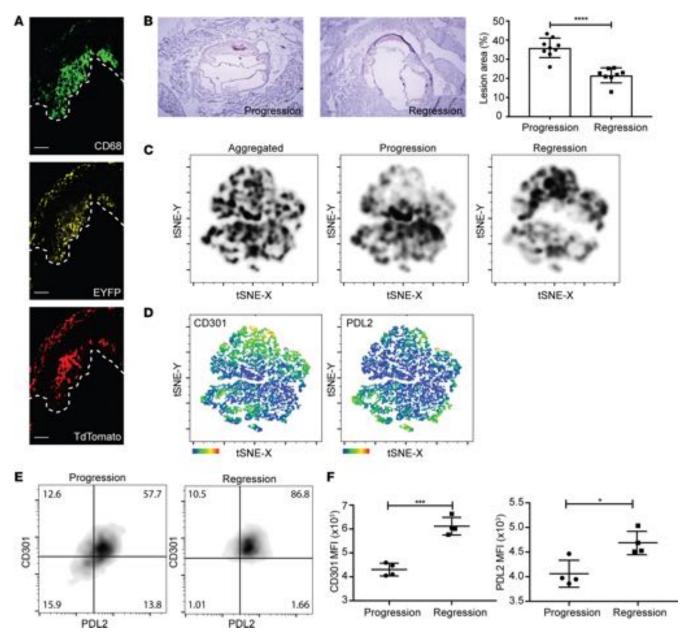


Figure 1. Fate mapping the conversion of CX3CR1⁺ cells into plaque macrophages. (A) Representative confocal images of aortic roots stained with CD68 (green), EYFP (yellow), and TdTomato (red) in BM chimeras of  $LdIr^{r/-}$  mice reconstituted with BM from  $Cx3cr1^{CreERT2-IRES-YFP/+}Rosa2E^{fl-tdTomato/+}$  mice (n = 3) gavaged with tamoxifen (TAM) at 14 and 15 weeks after feeding on a Western diet (WD) to label cells derived from CX3CR1⁺ monocytes. Scale bars: 50 μm. (B–F) Analysis of aortic arches by flow cytometry of  $Cx3cr1^{CreERT2-IRES-YFP/+}Rosa2E^{fl-tdTomato/+}$  mice injected with AAV-PCSK9 and fed WD for 18 weeks before TAM treatment. Progression group mice (n = 4) were then kept on WD, while regression group mice were switched to chow and treated with ApoB-ASO for 2 weeks (n = 4). (B) Representative bright-field images and quantification of lesion areas of aortic roots. Scale bars: 50 um. (C) Density plot of t-distributed stochastic neighbor embedding (t-SNE) analysis of CD11b⁺TdTomato⁺ cells from aortic arches of mice in progression and regression groups subjected to aortic digestion. The aortic arches were analyzed by flow cytometry for expression of PD-L2, CD301, EYFP, F4/80, and MHCII markers (n = 8). (D) Heatmaps of geometric mean fluorescence, (E) quadrant plots, and (F) geometric MFI of PD-L2 and CD301 expression on CD11b⁺TdTomato⁺ cells from progression and regression groups in atherosclerotic  $Cx3cr1^{CreERT2-IRES-YFP)+}Rosa2E^{fl-tdTomato/+}$  mice with AAV-mPCSK9 induction. Progression, n = 4-9; regression, n = 4-8. Statistical significance was calculated using Student's t test, and data are presented as mean  $\pm$  SEM (B and F). \*P < 0.05, \*\*\*P < 0.001, \*\*\*\*P < 0.0001.

reduces LDL production. AAVmPCSK9-treated *Cx3cr1*<sup>CreERT2-IRES-YFP/+</sup>*Rosa26*<sup>0-tdTomato/+</sup> mice were fed WD for 18 weeks, then treated with TAM. To compare atherosclerosis progression with regression, mice in the progression groups were kept on WD for an additional 2 weeks, whereas mice in the regression groups were switched to chow and treated with ApoB-ASO for 2 weeks to lower plasma LDL levels (Supplemental Figure 2A).



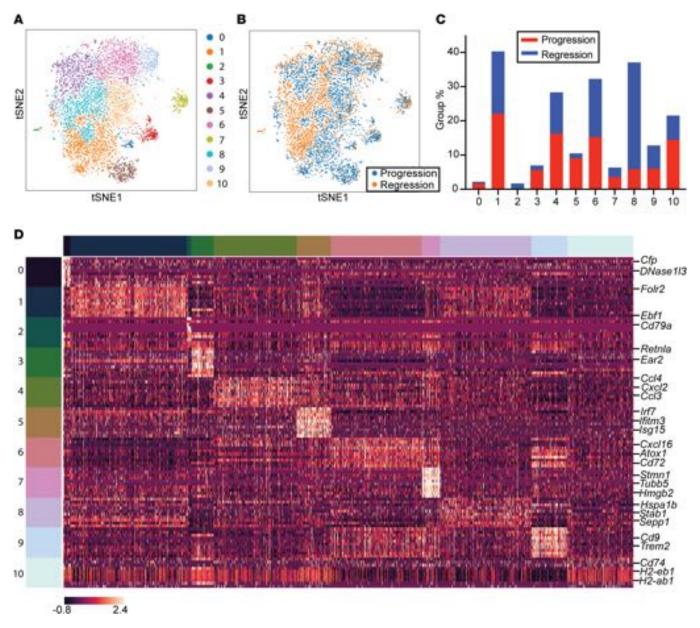


Figure 2. Heterogeneity of plaque macrophages derived from CX3CR1\* monocyte precursors in atherosclerosis progression and regression. Mice were treated as described in Figure 1. (**A** and **B**) We used Louvain clustering and multicore *t*-SNE to visualize 5355 CD11b\*TdTomato\* single cells isolated from aortic arches of Cx3cr1<sup>CreERT2-IRES-YFP/+</sup>Rosa26<sup>fl-tdTomato/+</sup> mice from the progression and regression groups (points, *n* = 4 mice per group; colored by [**A**] sub-cluster and [**B**] experimental group). (**C**) Cluster composition by percentages of experimental group (red, progression; blue, regression) in total sorted CD11b\*TdTomato\* cells. (**D**) Cell type signatures are shown in heatmap in the relative expression level. Row-wise *Z* score of ln(*X* + 1), where *X* denotes transcript count per cell after normalization (mean = 0, SD = 1); color scale of genes (rows) across cell clusters (columns) is shown.

We first confirmed that plaque areas were significantly decreased in the regression compared with the progression group (Figure 1B). We then performed FACS analysis to characterize TdTomato<sup>+</sup> cells from aortas (Supplemental Figure 2B). While the overall number of TdTomato<sup>+</sup> cells from aortas of mice undergoing progression and regression was not significantly different (Supplemental Figure 2C), there were phenotypic differences between the fate-mapped cells from the progression and regression groups, as visualized by *t*-distributed stochastic neighbor embedding (*t*-SNE) (Figure 1C). Notably, TdTomato<sup>+</sup> cells from the regression group expressed higher levels of PD-L2 and CD301 (Figure 1, D–F), which are both markers of alternatively activated M2 macrophages (12, 24).

Single-cell RNA-Seq analysis of aortic cells derived from CX3CR1<sup>+</sup> precursors in atherosclerosis progression and regression. To further define the molecular features associated with progression and regression in cells derived



from the CX3CR1<sup>+</sup> monocyte lineage, we FACS purified TdTomato<sup>+</sup> CD11b<sup>+</sup> cells from the aortic arches in the progression and regression groups. This allowed us to focus on cells belonging to the myeloid lineage by excluding T cells, NK cells, B cells, eosinophils, and neutrophils in the dump gate (Supplemental Figure 2B). We obtained between 1000 and 6400 TdTomato<sup>+</sup>CD11b<sup>+</sup> cells from each mouse and combined 4 samples for a total of 10,000–12,000 cells in each group. Single-cell RNA-Seq was performed on the 10x Genomics platform, which resulted in transcriptome profiles (after quality control filtering) of 3157 cells in the progression group and 2198 cells for the regression group. After data normalization, Louvain clustering of the aggregated data combined from the progression and regression groups identified 11 cell clusters, which were visualized using multicore *t*-SNE (Figure 2, A and B). Importantly, cells from all of the clusters expressed canonical myeloid cell, monocyte, and/or macrophage markers, including Csf1r, Cd14, Adgre1 (or F4/80), and Cd68 (Supplemental Figure 3A), indicating that we successfully excluded other hematopoietic cells that can express CX3CR1. The majority of the Louvain clusters are contiguous, which suggests a spectrum of related macrophage activation states, apart from the more distinct clusters 0, 3, 5, and 7 (Figure 2A) (discussed below).

Cluster 1 included the largest number of cells from both the progression and regression groups, at a relatively similar frequency, indicating a common feature of atherosclerosis (Figure 2C). Folate receptor β (Folr2) is the most differentially expressed gene in cluster 1 (herein referred to as "Folr2hi macrophages"; Figure 2D, Figure 3, and Supplemental Figure 3B). Expression of Folr2 was previously found to be increased in human atherosclerotic plaques, but not in normal arteries (25), and also to be expressed in a population of macrophages termed in a recent single-cell experiment as "resident-like macrophages" (18). Of special interest, the transcriptional profile of the cells in cluster 4 is dominated (Figure 2D, Figure 3, and Supplemental Figure 3B) by the expression of chemokines and cytokines (Ccl4, Cxcl2, Ccl3, Ccl2, Tnf, Cxcl1, Cxcl10, Ccl5), and appears similar to a population described as "inflammatory macrophages" by Cochain et al. (18) (herein referred to as "chemokinehi macrophages"). Clusters 6 and 9 are closely related to each other, with each having similar frequencies of cells in the progression and regression groups (Figure 2C). They share expression of Cxcl16, Atox1, CD72, Glipr1, but cluster 9 cells also express at higher levels of Trem2, Cd9, Lgals3, Spp1, Aldoa (Figure 2D, Figure 3, and Supplemental Figure 3B). Hence, cluster 9 is similar to the population of "TREM2hi macrophages" described in ref. 18 (herein referred to as "Trem-2hi macrophages"). Although clusters 6 and 9 are closely related, there are also distinct differences, for in cluster 6, the AA467197 gene (encoding normal mucosa of esophagus-specific gene 1 protein [NMES1]) is highly expressed (herein referred to as "NMES1hi macrophages").

These results indicate that the 3 macrophage populations described by Cochain et al (resident-like, inflammatory, and Trem2<sup>hi</sup>) are all derived from Cx3cr1<sup>+</sup> monocyte precursors, but they represent only a subset of the macrophage populations we isolated from the aortas. Additionally, since these macrophage populations are present in both progressing and regressing plaques, they represent general inflammatory features of atherosclerosis.

Transcriptional profile of cells more abundant during progression. We next focused on the cells in the distinct clusters 0 (1%), 3 (5%), 5 (9%), and 10 (14%) that contained CX3CR1+ cells mostly from the progression group (Figure 2C). Cluster 5 in particular expressed a strong type 1 IFN signature, including IFN regulatory factor 7 (IRF7) and IFN-stimulated gene 15 (ISG15), as well as myeloid cell nuclear differentiation antigen (MNDA) and IFN-induced transmembrane protein 3 (IFITM3) (herein referred to as "IFN signaturehi macrophages") (Figure 2D, Figure 3, and Supplemental Figure 4). Notably, type I IFN and myeloid type I IFN signaling has been shown to accelerate atherosclerosis progression in Ldlr-/- mice by promoting chemokine-dependent leukocyte recruitment (26). IFN signaturehi macrophages also exhibit increased expression of Ly6e and Ly6a, which encode for the Sca-1 antigen. With an anti–Sca-1 antibody, by FACS we can identify a distinct pattern of Sca-1 expression in a small subset of cells that are TdTomato+ (Supplemental Figure 6, A and B). Additionally, we verified that CD9 (enriched in Trem2hi macrophages) was highly expressed in a cluster of TdTomato+ cells (Supplemental Figure 6C). The CD9-expressing cells were clearly distinct from cells that express high MHC class II (MHCII) levels (Supplemental Figure 6D), representing the cluster of CD74hiMHCIIhi macrophages. Hence, we could verify several distinct cell clusters from TdTomato+ gated cells by antibody staining, as anticipated from the single-cell RNA-Seq results.

In cluster 0, expression of the endonuclease DNase113 is of interest and may also be associated with an IFN response (herein referred to as "DNase113hi macrophages"). However, genes expressed in cluster 3 were surprising, because Retnla and Ear2 are associated with exposure to type 2 cytokines such as IL-4 (herein referred to as "Retnlahi Ear2hi macrophages") (Figure 2D, Figure 3, and Supplemental Figure 4).



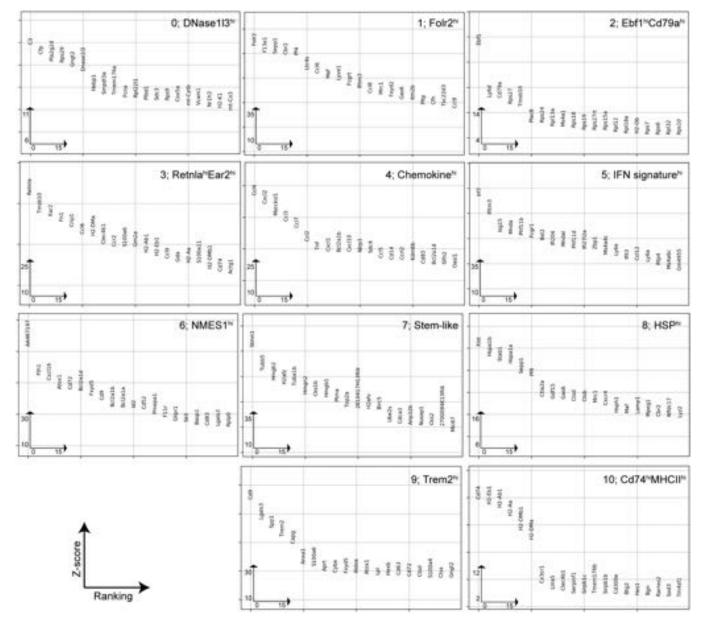


Figure 3. Classifications and gene lists of clusters of plaque macrophages derived from CX3CR1\* monocyte precursors in atherosclerosis progression and regression. Ranking of top 20 genes significantly overexpressed in each Louvain cluster, determined by statistical testing of one versus the rest with overestimated-variance t test. Mean of row-wise Z-score in the indicated cluster (y axis) is compared with bulk mean of Z-score for the ranking (x axis).

Hence, there is considerable heterogeneity of macrophage activation states under progression conditions, including the M1 features of a IFN type 1 signature, as well as the M2 features of an IL-4 signature. Our previous hypothesis that plaque progression is dominated by inflammatory M1 macrophages while plaque regression is dominated by M2 macrophages based on immunostaining and laser-capture plaque analyses (3, 15, 16) is likely too simplistic, as there was a clearly distinct IL-4 activated macrophage population (Retnlahi Ear2hi macrophages) in progressing plaques that was actually absent from regressing plaques. These data are more consistent with the concept of a spectrum of macrophage activation states (27–29) than a strict dichotomy between M1 and M2 macrophages.

Use of diffusion pseudotime (DPT) analysis can measure transitions in gene expression between cells, reconstruct cell developmental progression, and identify cell branching decisions and differentiation at a single cell level (30). Since CX3CR1 is downregulated as monocytes differentiate into macrophages, we chose from the cell population with highest average expression of CX3CR1 (cluster 7) (see below)



and then within this population, we chose cells with the highest individual CX3CR1 expression to be the "root" cell for DPT analysis to predict monocyte development (Figure 4, A and B). This approach identified RetnlahiEar2hi macrophages as having a trajectory separate from the main group comprising the other cell clusters (Figure 4, A and B), and these RetnlahiEar2hi macrophages were predominantly from progressing plaques (Figure 2, C and D, Figure 3, and Supplemental Figure 4). When we compared these data with our previous microarray data set (31), we noted that *Retnla* was also reduced in expression when comparing plaque regression with progression (Figure 4C). Since *Retnla* is highly expressed in macrophages exposed to IL-4, this initially surprising finding most likely reflects that compared with the regressing plaque, myeloid cells in the progressing plaque may be exposed to higher concentrations of both type 1 and type 2 cytokines.

It was striking that 3 of the distinct clusters of activated macrophages (DNase113hi macrophages, RetnlahiEar2hi macrophages, IFN signaturehi macrophages; Figure 2A) were highly enriched in cells from progressing plaques (Figure 2C), indicating that during progression, macrophages become more differentiated and activated than during plaque regression. This suggests that the environment of a regressing plaque for CX3CR1+ cells may be less complex. These clusters were not noted by the previous single-cell reports (17, 18), likely because we focused on cells derived from CX3CR1+ precursors and sampled approximately 5 times more cells.

Transcriptional profile of cells more abundant during regression. For the regression specific clusters, cluster 2 represented only a small fraction (<1%) of total cells, but was particularly interesting because of the high expression of B cell–associated genes, including the early B cell factor 1 (*EbfI*) and B cell antigen receptor complex-associated protein α chain (*Cd79a*) (herein referred to as "Ebf1hiCd79ahi macrophages"). Cluster 8 is an important contributor to regression (31%) and less so to progression (5%) (Figure 2C). Genes differentially expressed in this cluster include stabilin-1 (*Stab1*) and selenoprotein-1 (*Sepp1*), which are found in human and murine atherosclerotic plaques, with the former proposed to enhance efferocytosis and the latter to be antiinflammatory, both putative M2-associated functions (32). The heat shock protein (HSP) genes *Hspa1a* and *Hspa1b* were also differentially expressed in cluster 8 (Figure 2, C and D, Figure 3, and Supplemental Figure 4), indicating a protective role for HSPs in atherosclerosis regression (herein referred to as "HSPhi macrophages"). A protective role is also suggested by the reduced circulating HSP70-1 (*Hspa1a*) and HSP70-2 (*Hspalb*) associated with atherosclerosis progression and heart failure (33, 34).

Genes associated with cells from progression and regression states. During visualization by principal component analysis (PCA), we noticed a smooth transition between cells from the progression and regression groups along the principal component 1 (PC1) axis (Figure 4D). Genes with the largest-magnitude loading factors for PC1 (e.g., CD74 and MHCII molecules) were associated with cells in cluster 10 (Figure 2D, Figure 3, and Figure 4E), which contained cells more abundant in progression than in regression (herein referred to as "Cd74hiMHCIIhi macrophages").

Since we observed a smooth transition between cells from the progression and regression samples along the PC1 axis, we used the PC1 score as a "pseudotime" measure to determine the distributions of cells from the 2 sample groups. While overlapping, the distributions were clearly shifted (Figure 5A). We screened for genes whose cell-specific expression levels were correlated or anti-correlated with the cell-specific pseudotime values; this unsupervised analysis yielded 42 genes whose increased expression levels were associated with regression and 7 genes whose expression levels were associated with progression (Figure 5B). Among the 42 genes associated with regression, the analysis highlighted increased gene expression of *Cxcr4* (encoding CXC chemokine receptor type 4), *Wfdc17* (encoding WAP four-disulfide core domain 17), *Serpinb6a* (encoding serine peptidase inhibitor, clade B, member 6a), *Grn* (encoding granulin), *Ctsb* (encoding cathepsin B), and *Ctsd* (encoding cathepsin D), whereas increased expression of the genes *Cd74* (encoding cluster of differentiation 74), *H2-ab1*, *H2-eb1*, *H2-aa* (encoding MHCII molecules H2-Ab1, H2-Eb1, and H2-Aa), and *Malat1* (metastasis-associated lung adenocarcinoma transcript 1–long noncoding RNA) are associated with progression.

In terms of known expression or functions of these genes in atherosclerosis, increased CD74 expression is observed in human atherosclerotic plaques and *Malat1* is associated with atherosclerosis-related inflammation and lipid metabolism, although a distinct role for *Malat1* in atherosclerosis has not been described (35, 36). *Ctsb* and *Ctsd* (37) are associated with regression and have not been linked previously to M2 macrophage features (Figure 4E and Figure 5B).



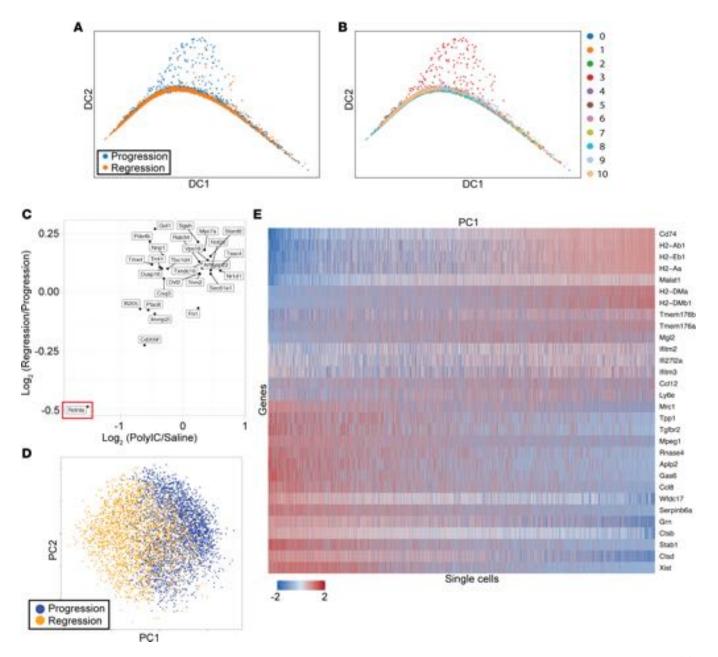


Figure 4. Diffusion pseudotime and principal component analysis identification of genes associated with atherosclerosis progression and regression. (A) Diffusion pseudotime (DPT) analysis identified a cellular branching point only present in the progression group (blue) for the Retnla<sup>hi</sup>Ear2<sup>hi</sup> cell cluster 3 (red). (B) The population with the highest expression of CX3CR1 was selected as the "root" cells to perform the DPT analysis to predict differentiation of CX3CR1\* cells. (C) Merged gene expression  $\log_2$  (polyl:C/saline) values from a previous atherosclerosis regression/progression study in Reversa mice (31) compared with gene-level  $\log_2$  (regression/progression) values from supervised analysis of the single-cell data for 27 differentially expressed genes, which confirms that *Retnla* expression (red box) is most negatively associated with regression in both data sets. (D) Principal component analysis (PCA) reveals a smooth transition of cells from progression and regression groups along the PC1 axis. (E) Heatmap of the genes with the greatest loading factors for the PC1 axis.

We also compared the gene-level pseudotime values with regression/progression expression ratios for differentially expressed genes in plaque CD68<sup>+</sup> cells measured in our previous study of an aortic transplant–based model of plaque regression (38). We found significant anticorrelation (Figure 5C) (P = 0.01, r = -0.35), as expected, since progressing cells have higher mean pseudotime than regressing cells (Figure 5A). Hence, these are features that are consistent between our previous studies and the current single-cell analysis.

Identification of a stem cell–like proliferative CX3CR1<sup>+</sup> cell cluster during atherosclerosis. As noted above, the population of cells in cluster 7 retained more CX3CR1 expression (Figure 6A). These cells also had a transcriptional profile very distinct from those of the other macrophage populations (Figure 3 and Supplemental Figure 4).



This population of cells was enriched for cell cycle genes (Figure 6B), similar to recent findings in cancer stem cells (39), indicating that they are highly proliferative (herein referred to as "stem-like macrophages"). Gene ontology analysis confirmed that "cell division" is an important pathway for this population of cells (Supplemental Figure 7). These cells also express monocyte and macrophage genes such as *Cd14*, *Adgre1*, *Csf1r*, and *Cd68* (Supplemental Figure 3A). We performed Ki-67 staining on plaque sections and confirmed that there were indeed CX3CR1-YFP+TdTomato+ cells expressing Ki-67 (Figure 6C).

We identified 138 genes that were expressed in these stem-like macrophages and compared the expression profile to cells in the ImmGen database. The expression profile of stem-like macrophages hierarchically clustered with myeloid progenitors (MLPs) and stem cells, indicating that they may have self-renewing properties consistent with the expression of cell cycle genes (Figure 6D and Supplemental Figure 5).

To confirm that the stem-like macrophages can be identified in an independent biological experiment, we next merged our data set to the recently published data set from Kim et al., in which the authors single-cell-profiled sorted CD45<sup>+</sup> cells isolated from whole aortas of *Ldlr*<sup>-/-</sup> mice fed a WD for 12 weeks (40). We performed MergeSeurat, aligned the data sets with canonical correlation analysis (CCA), and identified 11 clusters by Louvain clustering (Figure 7A). We then classified the cell populations with Single Cell Recognition (SingleR), a novel method for unbiased cell type recognition (41), and identified specific cell clusters as macrophages, monocytes, DCs, and innate lymphoid cells (ILCs) (Figure 7B). Most of the cells in the merged data set were classified as macrophages by SingleR, and we identified a distinct macrophage cluster (cluster 6) that expressed the highest level of cell cycle genes and thus exhibited stem-like properties (Figure 7, B–E). Notably, this cell cluster was present in a similar proportion (3.4%) in total sequenced cells (Figure 7D) from each data set (i.e., both progression and regression samples and Kim et al.'s data set), demonstrating that the abundance of this cell cluster is consistent in the different atherosclerosis models and settings. We also confirmed that most of the cells in this cluster maintained high expression of CX3CR1 (Figure 7F).

Hence, we identify a population of proliferating cells with a stem cell–like signature derived from CX3CR1<sup>+</sup> precursors that are present in both atherosclerosis progression and regression models, as well as in an independent *Ldlr*<sup>-/-</sup> mouse atherosclerosis experiment (40). This finding extends the current view that tissue-resident macrophages can proliferate in plaques (42).

#### Discussion

The above results represent the introduction of single-cell RNA-Seq combined with genetic fate mapping to track the cellular states during the differentiation of CX3CR1+ cells into macrophages in atherosclerotic plaques during their progression and regression. In addition to confirming the considerable heterogeneity of macrophages in plaques reported by others (e.g., refs. 43–46), our approach has led to several observations: (i) atherosclerosis progression is associated with differentiation into more distinct macrophage states than during regression; (ii) the spectrum of macrophage activation states in both progressing and regressing plaques has greater complexity than the traditional definition of the M1 and M2 polarization phenotypes; and (iii) there is a cluster of proliferating CX3CR1+ monocytes with a stem cell-like signature in both progressing and regressing plaques.

Our first observation is supported in the simplest sense by identification of 4 cell clusters (DNase113hi, RetnlahiEar2hi, IFN signaturehi, Cd74hiMHCIIhi macrophages) enriched from progressing plaques versus 2 cell clusters (Ebf1hiCd79ahi, HSPhi macrophages) enriched from regressing plaques (Figure 2C). In addition, pseudotime analysis suggested additional lineage branching for cells enriched in progression (RetnlahiEar2hi macrophage) rather than regression. We also found that 3 of the cell clusters enriched in progressing plaques (DNase113hi, RetnlahiEar2hi, IFN signaturehi macrophages) were more transcriptionally distinct (i.e., more separated from the main macrophage cluster/cloud) from the other macrophage populations. This indicates that during progression, macrophages may become more differentiated and activated than during plaque regression. Notably, these distinct clusters were not reported by the previous single-cell reports, but embedded in our data are the 3 macrophage populations described by Cochain et al.: resident-like, inflammatory, and Trem2hi macrophages (Folr2hi, chemokinehi, and Trem2hi macrophages, respectively in our data). Hence, these 3 macrophage populations noted previously are derived from CX3CR1+ monocyte precursors but only represent a fraction of different macrophage activation states present in atherosclerotic aortas, which we were able to achieve by sampling approximately 5 times more cells and focusing our analysis only on cells derived from CX3CR1+ precursors. In studies combining cytometry by time of flight (CyTOF) (mass cytometry)



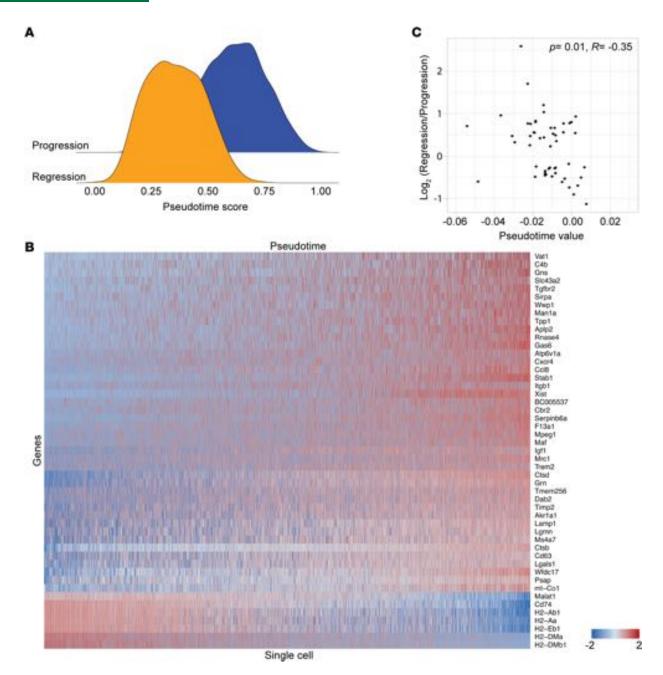


Figure 5. Pseudotime scores and related genes associated with atherosclerosis progression and regression. (A) We used PC1 scores as a pseudotime measure and show shifted distributions of progression and regression cells. (B) Heatmap of genes identified to be significantly correlated with the pseudotime score. (C) We identified 53 genes significantly correlated with the pseudotime score that are negatively associated (r = -0.347, P = 0.01103) with merged gene expression log, (polyl:C/saline) values from a previous atherosclerosis regression/progression study (38).

with single-cell RNA-Seq, Winkels et al. found evidence to suggest that leukocyte heterogeneity is lower in healthy (vs. atherosclerotic) aortas (17). Our finding that macrophages derived from CX3CR1<sup>+</sup> cells are less complex in regressing plaques is consistent with this suggestion, as during regression, the plaque microenvironment is improved by the reversal of hyperlipidemia. Alternatively, macrophages in progression could have more distinct activation states because of stronger environmental stimuli.

The greater activation of macrophages derived from CX3CR1<sup>+</sup> cells in progressing plaques is consistent with atherosclerosis being an inflammatory disease. Even more interesting, perhaps, are the data concerning the spectrum of macrophage activation states in progressing and regressing plaques. While macrophages characteristic of the M1 (classically activated) and M2 (alternatively activated, inflammation resolving) polarization extremes have been found by immunohistochemical markers in human and murine plaques (47), only recently



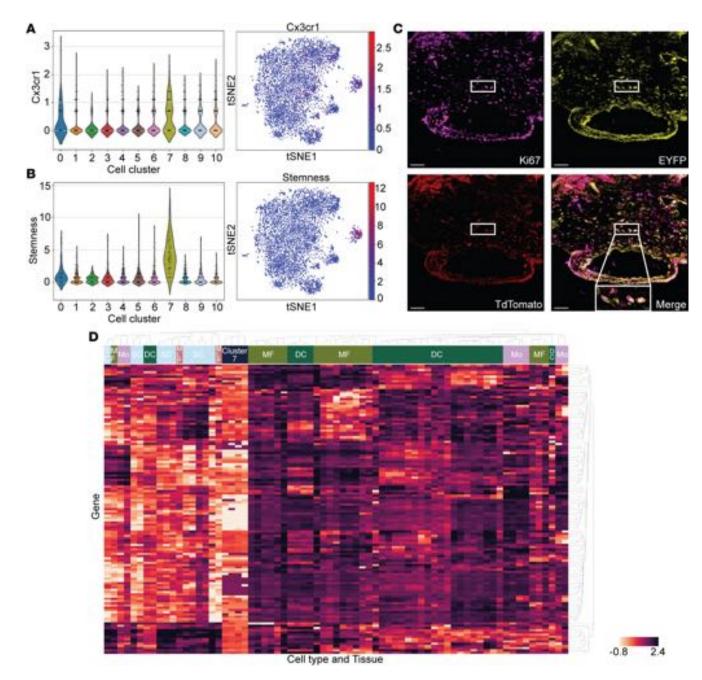


Figure 6. Identification of a proliferative "stem-like" cell cluster that retains CX3CR1 expression. (A) Violin plot and heatmap showing higher expression of CX3CR1 in cluster 7. (B) Violin plot and heatmap showing higher expression of cell cycle genes in cluster 7. (C) Representative immunofluorescence images of aortic root staining with Ki-67 (purple), EYFP (yellow), and TdTomato (red) from the Cx3cr1creERT2-IRES-YFP/+Rosa26<sup>fl-tdTomato/+</sup> atherosclerotic mice in the progression group. Scale bars: 50 µm. (D) 138 genes that were expressed more than one read in 90% of cells in cluster 7 were compared with macrophages, monocytes, myeloid progenitors, stem cells, and DCs extracted from the ImmGen database. Cells from cluster 7 were randomly grouped into 4 subgroups (shown in black), and the median expression of each gene is shown. Heatmap analysis illustrates the clustering of similar cell types and genes based on normalized gene expression profiles.

have dynamic changes in marker expression been described. As we first reported in transcriptome analyses of macrophages (CD68<sup>+</sup> cells) from progressing and regressing plaques, there was an obvious enrichment in cells expressing arginase I and other markers associated with the M2 state (38). The origin of these cells was from circulating Ly6C<sup>hi</sup> monocytes, and if STAT6-dependent M2-associated polarization was prevented, M2-marker expression enrichment and, importantly, plaque inflammation resolution, were blocked (14).

Thus, the simple scenario based on those studies is that when the environment of an atherosclerotic plaque became healthier, newly recruited monocytes skewed away from the M1 toward the M2 direction



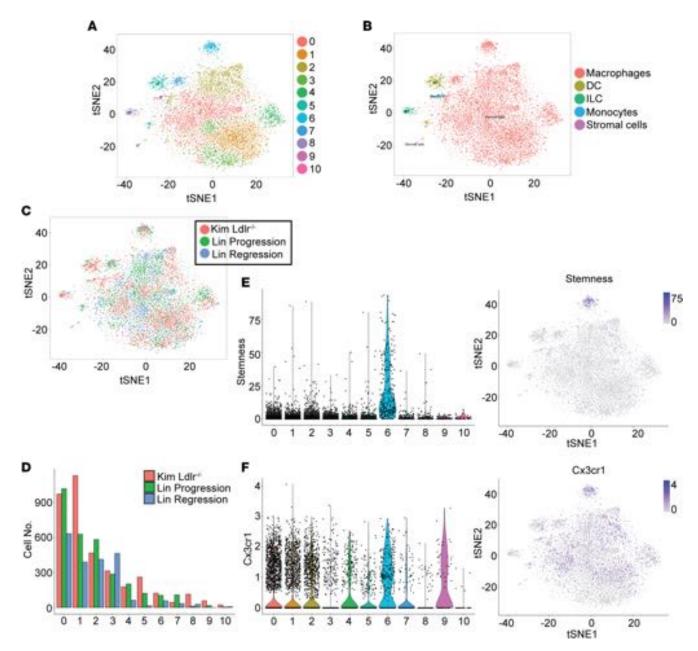


Figure 7. Validation of the presence of a "stem-like" cell cluster from an independent study of atherosclerotic mice. A recently published single-cell data set from Kim et al. (ref. 40; "Kim") from sorted CD45\* cells isolated from whole aortas of Ldlr-/- mice fed WD for 12 weeks was combined with our progression and regression data sets (Lin Progression, Lin Regression) with Seurat merging and aligned with canonical correlation analysis (CCA). (A) t-SNE visualization of the 8906 single cells from both data sets after Louvain clustering and colored by sub-cluster.(B) SingleR method was used for unbiased cell classifications of each sub-cluster against the ImmGen database and colored and labeled accordingly on the t-SNE plot. (C) t-SNE plot colored based on experimental group and data sets, showing that cluster 6 includes cells from all 3 experiments. (D) Cluster composition by cell numbers of experimental groups and data sets (red, Kim Ldlr-/-; green, Lin Progression; blue, Lin Regression) in total single cells. (E) Violin plot and heatmap showing the highest expression of cell cycle ("Stemness") genes in cluster 6. (F) Violin plot and heatmap showing the expression of CX3CR1 in cluster 6.

of polarization, resolved the inflammation, and promoted beneficial remodeling of the artery. The present results, using a more incisive approach than previously possible, indicate that there is a diverse spectrum of macrophage activation states in atherosclerosis progression and regression, and the previous association of M1 macrophages only with progression and of M2 macrophages only with regression may be overly simplistic. While macrophages with M2 features (cell surface expression of PD-L2 and CD301 and increased MRC1 expression) were more abundant during regression (Figure 1, D–F, Figure 2, and Figure 3), a distinct cluster of macrophages (Retnla<sup>hi</sup>Ear2<sup>hi</sup> macrophages) expressing M2 signature genes was present in



progression, and not regression (Figures 2 and 3). We should note that a broad distribution of M1- and M2-associated gene expression was also found in the macrophage populations classified by Cochain et al., with many displaying heterogeneous patterns within and overlaps between populations (18).

Turning to our third observation, monocytes are known to constitutively enter non-lymphoid organs and recirculate to lymph nodes in the steady state without differentiation to macrophages or DCs (48). These Ly6ChiMHCII+ monocytes may play a role in antigen presentation or surveillance of inflamed or steady-state tissue (48–50). In the present studies, we identified a distinct cluster of cells (stem-like macrophages) with proliferative features that maintain CX3CR1 expression as well as markers of monocytes and macrophages, while sharing transcriptional profiles with stem cells. Macrophage proliferation in progressing plaques in *Apoe*-/- mice and other models has been described (ref. 42; reviewed in ref. 6). The present results are also consistent with our finding in regressing plaques of a small population of cells positive for the proliferation marker Ki-67 (14).

The source of the proliferating macrophages could be either tissue-resident macrophages, inflammatory monocyte-derived macrophages (12) or even the proliferative CX3CR1<sup>+</sup> monocytes that we have now identified in both progressing and regressing plaques (Figures 2 and 6). However, this cell cluster is relatively rare (Figure 2C), and the roles or fate of these proliferative tissue-resident monocytes remains to be elucidated. Nonetheless, uncovering this population raises the possibility that there are proliferating stem cell-like monocytes that can self-renew within inflamed tissues, serving as an additional reservoir for tissue macrophages that adopt different activation states depending on the microenvironments of atherosclerosis (Supplemental Figure 8).

In conclusion, we have significantly extended studies of atherosclerosis by a combination of a state-of-the art mouse model, fate mapping, transcriptomics, and bioinformatics. In addition to analyzing many more cells in progressing plaques compared with other studies (17, 18), we have obtained new data on regressing plaques. As a result, we have identified multiple new cellular phenotypes, with distinct molecular features, that are enriched in populations of macrophages in atherosclerosis progression and regression. We also found a number of macrophage populations with features that are shared between these disease states, which highlights the possibility that therapies based on data from progressing plaques alone may have unintended adverse consequences for the regression process. Further progress will be needed, therefore, to achieve the goal of identifying the targets with the best likelihood of therapeutic success by avoiding this pitfall.

#### Methods

See the Supplemental Methods for a detailed explanation of all experimental procedures.

Study approval. All animal procedures were approved by the NYU School of Medicine IACUC.

Statistics. All data are presented as mean  $\pm$  SEM. Two-tailed unpaired Student's t test was used calculated with Prism 7.0 from GraphPad. P < 0.05 was considered significant.

Data availability. The full data set is available in the NCBI's Gene Expression Omnibus (GEO GSE123587).

#### **Author contributions**

JDL, EAF, and PL designed experiments and wrote the manuscript. JDL, HN, CM, KR, STY, NV, and AW performed experiments. JP, XN, SAR, and EJB analyzed single-cell RNA-Seq data.

#### **Acknowledgments**

This article is dedicated to the memory of co-author Hitoo Nishi, who died at age 42 in August 2018. His initial studies inspired our taking new directions in our research, with this article containing his critical contributions. He was an exceptional person both professionally and personally. We acknowledge Yutong Zhang, Karina Ray, and Adriana Heguy at the NYU Langone Genome Technology Core for help with single-cell RNA-Seq. The NYU Langone Genome Technology Center is partially supported by Cancer Center Support Grant P30CA016087 at the Laura and Isaac Perlmutter Cancer Center. We also acknowledge the Microscopy Laboratory at NYU School of Medicine for providing equipments for confocal imaging. PL and EAF were supported by grants from the NIH (HL084312, AI133977) and the Department of Defense (W81XWH-16-1-0255, W81XWH-16-1-0256).



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### Review

#### **HDL** and Reverse Cholesterol Transport

#### Basic Mechanisms and Their Roles in Vascular Health and Disease

Mireille Ouimet, Tessa J. Barrett, Edward A. Fisher

Abstract: Cardiovascular disease, with atherosclerosis as the major underlying factor, remains the leading cause of death worldwide. It is well established that cholesterol ester-enriched foam cells are the hallmark of atherosclerotic plaques. Multiple lines of evidence support that enhancing foam cell cholesterol efflux by HDL (high-density lipoprotein) particles, the first step of reverse cholesterol transport (RCT), is a promising antiatherogenic strategy. Yet, excitement towards the therapeutic potential of manipulating RCT for the treatment of cardiovascular disease has faded because of the lack of the association between cardiovascular disease risk and what was typically measured in intervention trials, namely HDL cholesterol, which has an inconsistent relationship to HDL function and RCT. In this review, we will summarize some of the potential reasons for this inconsistency, update the mechanisms of RCT, and highlight conditions in which impaired HDL function or RCT contributes to vascular disease. On balance, the evidence still argues for further research to better understand how HDL functionality contributes to RCT to develop prevention and treatment strategies to reduce the risk of cardiovascular disease. (Circ Res. 2019;124:1505-1518. DOI: 10.1161/CIRCRESAHA.119.312617.)

**Key Words:** atherosclerosis ■ cardiovascular diseases ■ cholesterol ■ diabetes mellitus ■ foam cells ■ reverse cholesterol transport

The Framingham Heart Study in the 1960s was the first 1 study to report inverse associations between cardiovascular risk and plasma HDL-C (high-density lipoprotein cholesterol). This landmark discovery inspired investigations into the mechanisms by which HDL confers atheroprotection, leading to the identification of the reverse cholesterol transport (RCT) pathway.<sup>2</sup> RCT is defined as the process by which cholesterol moves out of cells in peripheral tissues (including foam cells in atherosclerotic plaques), enters the circulation, and is excreted in the feces. HDL's cardiovascular protective effect has conventionally been attributed to its ability to act as both the acceptor of cholesterol from cells and as the cholesterol carrier in the RCT pathway, including delivery to the liver. It has been estimated from observational studies that cardiovascular risk decreases by ≈2% to 3% per 1-mg/dL increase in HDL-C.3 Implicit in this view is that the level of HDL-C in the plasma is a faithful biomarker of the ability of the HDL particles to mediate RCT. In 2019, this is now recognized to be an oversimplification as HDL-C measurements do not necessarily reflect either the overall abundance of HDL particles, the distribution of HDL subspecies, 4 or RCT capacity.5 Additionally, data from human genetic studies6 and a host of negative HDL-raising clinical trials have led to much controversy over the HDL hypothesis. This controversy, however, should not negate the strong experimental evidence that a major function of HDL particles is to mediate RCT and that an increased understanding of the mechanisms by which this is accomplished represents a chance to revise and refine the

HDL hypothesis. The framework of this review is illustrated in the Figure, with the points made in the legend discussed in detail below.

#### **Cholesterol Efflux and Peripheral Cells**

All mammalian cells require cholesterol, with the highest concentration in the plasma membrane and the lowest in the endoplasmic reticulum (ER) membrane. The amount of free cholesterol is maintained in a relatively narrow range, making cellular cholesterol homeostasis essential for normal cell function. Based on pioneering data from many laboratories, <sup>2,7</sup> RCT came to be described as the process by which HDL acts as the specific cholesterol acceptor that transports excess cholesterol stores within peripheral tissues to the plasma, and then delivers it to the liver, where it can be directly excreted into the bile or be metabolized into bile acids/salts before excretion. These studies naturally stimulated much research in a variety of in vitro systems, to investigate at the cellular level the initial step of RCT. Herein, we will focus on the roles of macrophages and vascular smooth muscle cells (VSMCs) in the early steps of the RCT process, given their crucial role in the development of cardiovascular diseases (CVDs), especially atherosclerosis. We will also discuss other aspects of the RCT pathway, including its quantitative assessment in vitro and in vivo.

#### **Macrophages**

Macrophage extracellular cholesterol is primarily derived from the internalization of plasma lipoproteins or from the

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#### **Nonstandard Abbreviations and Acronyms**

ABCA1/G1 ATP-binding cassette proteins A1 and G1 ACAT acyl-CoA:cholesterol acyltransferase **AGEs** advanced glycation endproducts CAD coronary artery disease CCR7 C-C chemokine receptor type 7 CD cyclodextrin

CE cholesteryl ester CEC cholesterol efflux capacity **CETP** cholesteryl ester transfer protein CVD cardiovascular disease ER endoplasmic reticulum FXR farnesoid X receptor

HDL-C high-density lipoprotein cholesterol

KLF4 Kruppel-like factor 4 LAL lysosomal acid lipase

**LCAT** lecithin:cholesterol acyltransferase LDL-C low-density lipoprotein cholesterol LDLR low-density lipoprotein receptor

LDs lipid droplets **LXRs** liver X receptors NPC Niemann-Pick Type C **ORPs** oxysterol-binding related proteins **OSBP** oxysterol-binding proteins

**RAGE** receptor for advanced glycation endproducts

**RCT** reverse cholesterol transport SR-B1 scavenger receptor class B type 1 **SREBP** sterol regulatory element-binding protein

**SRF** serum response factor

**StAR** steroidogenic acute regulatory protein TICE transintestinal cholesterol efflux **VLDL** very-low-density lipoprotein **VSMCs** vascular smooth muscle cells

efferocytosis of apoptotic cells, which enter the cellular pool together with newly synthesized cholesterol. To prevent toxicity, surplus cholesterol is effluxed from the cells to extracellular acceptors or converted to cholesteryl ester (CE) and stored in cytosolic lipid droplets (LDs). There are many mechanisms by which cells are defended against cholesterol toxicity. For example, the LXRs (liver X receptors), key sterol-sensitive transcription factors in macrophages that regulate intracellular cholesterol (reviewed in Hong and Tontonoz<sup>8</sup>), are induced by excess cholesterol. LXRs, in turn, drive the expression of numerous genes within the efflux pathway, including ABCA1/ G1 (ATP-binding cassette proteins A1 and G1), which are key cellular cholesterol transporters. Adding to the effort to reduce cellular sterol content, LXR also controls the expression of the inducible degrader of the LDLR (low-density lipoprotein receptor; IDOL), an E3 ubiquitin ligase that promotes the degradation of the LDLR. This limits further uptake of exogenous cholesterol via LDLR.9 Another defensive response to elevated cell cholesterol is the inhibition of the processing of the SREBP (sterol regulatory element-binding protein), leading to decreased expression of genes that regulate cholesterol

synthesis (HMGCR) and uptake (LDLR). Yet, another consequence of cellular cholesterol excess is the reduced expression of a small microRNA encoded in SREBP's intronic region, miR-33, which among its targets of translational repression are the mRNAs encoding numerous factors in the RCT pathway (ABCA1, NPC [Niemann-Pick Type C]-1, ABC11, and ATP8B1).10-12 miR-33a is encoded in the intron of the SREBP-2 gene in mice and humans, while its isoform miR-33b is encoded within the SREBP-1 gene in higher mammals. 12 Notably, inhibition of miR-33 in mice and nonhuman primates holds therapeutic promise as it has been shown to enhance RCT, 12-14 protect against atherosclerosis 15-17 and promote atherosclerosis regression, 14,18 though some controversy surrounds its role in regulating hepatic triglyceride and fatty acid metabolism. 19-22 A recent report by the Fernandez-Hernando group demonstrates that repression of ABCA1 is the primary mechanism by which miR-33 regulates macrophage cholesterol efflux and atherogenesis.<sup>23</sup> Although miR-33 is the most characterized of the miRNAs that regulate RCT, there are at least 10 others that also have targets in this pathway (reviewed in Feinberg and Moore<sup>24</sup>).

ABCA1 and ABCG1 are critical receptors for the initial step of RCT in atherosclerotic plaques, that is, cholesterol efflux out of foam cells.<sup>25-28</sup> Foam cells are traditionally thought to be cholesterol-laden macrophages originating from monocytes, but as reviewed below, they can also be macrophagelike cells originating from cholesterol-laden VSMCs.29-33 Before efflux, cholesterol must be in its free (unesterified) form to be pumped out of cells. This is the case in vitro and in vivo, including in atherosclerosis, where the rate-limiting step in RCT is hydrolysis of LDs in vascular foam cells to generate free cholesterol for efflux.34-36 Consistent with this are several studies reporting that stimulation or inhibition of macrophage foam cell CE hydrolysis regulates RCT and atherosclerosis.<sup>37-41</sup> LD cholesterol undergoes constitutive cycles of hydrolysis and re-esterification. Free cholesterol released from LDs via CE hydrolysis can either traffic to the plasma membrane and be effluxed to a cholesterol acceptor, or, in a futile cycle be re-esterified by the ER-resident protein ACAT (acyl-CoA:cholesterol acyltransferase).42,43 Original studies by Brown and Goldstein characterizing this futile cycle indicated that cytoplasmic CE hydrolysis in macrophage foam cells was mediated by extra-lysosomal, cytoplasmic neutral CE hydrolases. 42,44 Nevertheless, knocking down or out potential CE hydrolases in macrophages never entirely abolishes cellular CE hydrolysis.36 The importance of addressing the missing regulators of CE hydrolysis is underscored by the many studies to date showing that increasing the hydrolysis of LD CE increases cholesterol efflux and is antiatherogenic.36

A key insight into CE hydrolysis came from the observation that the loading of macrophages with proatherogenic lipoproteins can activate autophagy, promote sequestration and delivery of LDs to lysosomes for degradation, and enhance RCT from macrophage foam cells.<sup>45</sup> Autophagy is a ubiquitous cellular process by which cytoplasmic components are degraded within lysosomes. There are 3 types of autophagy in mammalian cells: (1) macroautophagy, where cargo is sequestered in de novo formed autophagosomes that subsequently fuse with lysosomes, (2) microautophagy, where cargo is taken

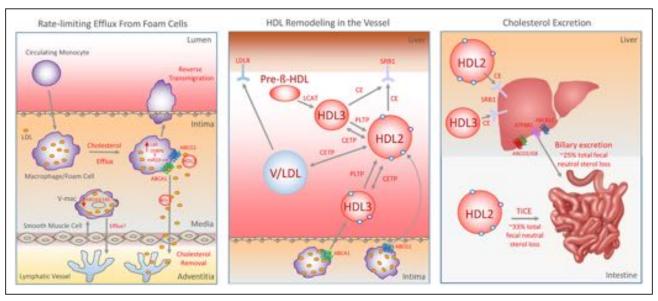


Figure. Key steps of reverse cholesterol transport (RCT). RCT begins with the removal of cholesterol from arterial foam cells that are of vascular smooth muscle cell (V-mac) or macrophage origin (left). This is the rate-limiting step of the RCT pathway and requires the efflux of free cholesterol to cholesterol acceptors such as nascent or mature HDL (high-density lipoprotein) along with macrophage egress from the plaques. While RCT from macrophage foam cells requires the cholesterol pumps ABCA1 and ABCG1 (ATP-binding cassette proteins A1 and G1), mechanisms regulating RCT from intimal vascular smooth muscle cells that have transdifferentiated to macrophage-like foam cells (V-mac) are not well understood, though cholesterol efflux from V-macs appears defective relative to macrophage foam cells. Intimal-derived HDL cholesterol can reach the liver 1 directly through binding the hepatic HDL receptor SR-B1 (scavenger receptor class B type 1) that selectively removes cholesteryl ester (CE) from HDL2 and HDL3 or 2 indirectly via apoB-containing lipoproteins (VLDL [very-low-density lipoprotein] or LDL [V/LDL])—to which cholesterol is transferred by the action of CETP (cholesterol ester transfer protein)—that are cleared by hepatic LDLR (middle). PLTP (phospholipid transfer protein) also plays an important role in regulating HDL metabolism through HDL remodeling. Finally, the last step of the RCT pathway is cholesterol excretion into the feces (right). This can occur through biliary cholesterol excretion or transintestinal cholesterol efflux (TICE) that mediate ≈25% and 33% of total fecal neutral sterol loss, respectively. LCAT indicates lecithin:cholesterol acyltransferase; LXR, liver X receptors; and OSBP, oxysterol-binding protein.

into lysosomes by invagination and pinching of the lysosomal membrane into the lysosome lumen, and (3) chaperonemediated autophagy, where single proteins are recognized by chaperones and delivered to lysosomes via a membrane translocation complex. 46 Macroautophagy—referred to as autophagy hereafter—is the subtype most relevant to this review and can sequester cytosol in bulk or selectively. This pathway depends on numerous autophagy proteins that organize into functional complexes that orchestrate the autophagic process, first generating the limiting membranes or phagophores that elongate in cup-shaped structures that engulf cytoplasmic cargo, fusing to become autophagosomes.<sup>47</sup> Subsequent autophagosome fusion with lysosomes releases the autophagic body, or in the case of RCT—LDs, into the lysosome lumen where they are degraded. LAL (Lysosomal acid lipase), encoded by the LIPA gene in humans, is responsible for the hydrolysis of LD-associated CE to generate free cholesterol for efflux. 45 Genome-wide association studies have identified several loss of function mutations in LIPA as causative of Wolman disease, cholesterol ester storage disease, and coronary artery disease (CAD).48

Targeted LD degradation by autophagy, or lipophagy, represents a novel pathway to regulate RCT, and it is thought that enhancing autophagy holds promise to promote lipid clearance from the atherosclerotic vascular wall. In particular, atherosclerosis development is associated with a progressive defect in autophagy in cells positive for macrophage markers MOMA-2 (monocyte/macrophage antibody) and CD11b in the plaque,<sup>49</sup> and defective clearance of cargo tagged by the autophagy marker p62/SQSTM1 is readily observed

by detection of its accumulation in whole aortic protein lysates. 49.50 Further, inhibition of autophagy pathways in mice promotes atherosclerosis development by reduced lipophagy and lysosome-mediated cholesterol cellular efflux, which contributes to inflammasome hyperactivation, elevated cell death, and defective efferocytosis within plaques. 36,49,51 A critical role for autophagy and lysosomal biogenesis to suppress atherosclerosis development is supported by studies showing that systemic miR-33 inhibition or macrophage overexpression of the master transcriptional regulator of autophagy and lysosomal genes, TFEB (transcription factor EB), restores plaque macrophage autophagy, improves efferocytosis and inflammation, and ultimately reduces atherosclerosis burden. 50,52

The routes by which free cholesterol generated at the site of lipid lipolysis (within lysosomes or at the LD surface) reaches the ABCA1 and ABCG1 cholesterol transporters on the plasma membrane depends on both vesicular and nonvesicular trafficking pathways, although the precise mechanisms are poorly characterized.<sup>53,54</sup> The general working hypothesis is that cholesterol transporters sit at the plasma membrane and await delivery of cholesterol to be effluxed; but, this is an oversimplification as these can be motile, as exemplified by ABCA1 that continuously shuttles between the plasma membrane and endolysosomal compartments.<sup>55</sup> This shuttling is a regulated process that is impeded by hypoxia. 56,57 ABCG1 relocalizes from the Golgi and ER to the plasma membrane following LXR activation to stimulate efflux to HDL.<sup>58</sup> This involves ABCG1 concentrating on intracellular endocytic vesicles (eg, recycling endosomes) to apparently

redistribute sterols to the plasma membrane outer leaflet on fusion, so that cholesterol desorbs to exogenous lipid acceptors such as HDL.59 Transporters that possess distinct subcellular localizations likely preferentially efflux cholesterol from specific intracellular pools; for instance, apoA-I/ABCA1 retroendocytosis is required for efficient cholesterol efflux under lipid-loaded conditions<sup>60</sup> and conversely, ABCA1-mediated cholesterol efflux is primarily dependent on autophagy for its cholesterol source.45

Another cholesterol trafficking pathway is mediated by OSBP (oxysterol-binding)-ORPs (related proteins). They constitute a family of lipid binding/transfer proteins that can facilitate nonvesicular transfer of cholesterol between lipid bilayers, increasing the efficiency of cholesterol transport between subcellular membranous organelles. Roles for many of the ORPs within this family (12 members in total) as sterol sensors or transporters at distinct subcellular sites have been recently reviewed.<sup>61</sup> Recently, ORP6 was found to regulate cholesterol efflux and HDL homeostasis, suggesting that it may represent a novel regulator of the RCT pathway,62 yet mechanisms by which ORP6 and other ORP members may regulate this pathway are poorly understood. Other key mediators of interorganelle lipid trafficking that may represent potential therapeutic enhancers of RCT include the soluble lipid transfer proteins StAR (steroidogenic acute regulatory protein) D4, MLN64, and NPC proteins.63 More recently, Aster proteins have emerged as novel mediators of nonvesicular cholesterol transport at contact sites between the plasma membrane and ER, providing a new mechanism by which HDL-derived cholesterol can be mobilized through the selective HDL cholesterol uptake pathway.<sup>64</sup>

#### **Vascular Smooth Muscle Cells**

While much of the focus on the early steps of RCT has been on defining mechanisms of efflux from macrophages, there have also been investigations on VSMCs. VSMC plasticity in atherosclerosis is well recognized; for example, in the media of atherosclerotic arteries, they are considered to be contractile and can become proliferative, migrate to the intima, where they are synthetic, and exhibit the loss of many markers of the VSMC in the contractile state, such as smooth muscle cell actin and myosin heavy chain.30 Though it has been long appreciated that VSMC in the intima can also take up cholesterol through a variety of pathways, 29,65,66 phenotypic changes in these VSMC-foam cells at the molecular level had not been systematically studied. In 2003, it was shown that loading mouse primary VSMC with cholesterol in vitro resulted in the concurrent loss of VSMC marker expression and the gain of macrophage-associated gene expression.67

One implication of these findings is that conventional histological markers used to identify macrophages in plaques would include cells of VSMC lineage. Three studies using lineage-marking approaches in mice<sup>31,32,68</sup> and a variety of assays for human plaques<sup>69</sup> were published in quick succession to confirm that, indeed, there are macrophage-appearing cells of VSMC origin in human and mouse plaques. These results have also been replicated in studies examining the clonality of VSMC in atherosclerotic plaques in mice. 70,71 The percent of the macrophage-marker positive cells of VSMC origin varied among the studies, but it was substantial in all, ranging from 30% to 70% (increasing with the stage of disease). The molecular regulation of this transition process has been studied mainly in vitro, where it was shown that cholesterol loading suppresses miR-143/145, resulting in reduced expression of the canonical VSMC regulatory transcription factors, SRF (serum response factor) and myocardin. Suppression of miR-143/145 also increased levels of KLF4 (Kruppel-like factor 4), a regulator of macrophage gene expression. 72 Consistent with the in vitro implication of KLF4 in the acquisition of macrophage features is the work from the Owens lab, which showed that in VSMC-specific KLF4-deficient mice, the percentage of macrophage-like cells derived from VSMC in atherosclerotic plaques was ≈50% versus those in control mice.<sup>32</sup> A recent study has also implicated integrin β3 in the transition of VSMC to macrophage-like cells in mouse atherosclerotic plaques.71

The relevance of cholesterol efflux to the macrophage-like transition of VSMC is suggested by the results in vitro that by providing cholesterol acceptors (HDL or apoA-I) to cholesterol-loaded cells, this completely reversed their macrophagelike phenotypes to the preloaded VSMC state.<sup>31</sup> Evidence that impaired efflux may be operating in vivo, and thereby sustaining the effects of cholesterol loading, comes from 2 strands of evidence. First, as Choi et al<sup>73</sup> have shown, ABCA1 expression is reduced in intimal-like VSMC (derived from arteries of Wistar-Kyoto rats) and in vitro, these cells exhibit less binding of apoA-I compared with those isolated from the medial layer. Similarly, ABCA1 expression was found to be low in human intimal VSMC, more so in advanced relative to early atherosclerosis.<sup>69</sup> Recently, CD45<sup>-</sup> cells (presumably VSMCderived) from ApoE<sup>-/-</sup> mice were also found to exhibit reduced ABCA1 expression relative to CD45<sup>+</sup> (presumably monocytederived) foam cells.<sup>33</sup>

Such changes would be likely to make cholesterol taken up by intimal VSMC linger and not readily enter the RCT pathway. It should be noted, however, that in other studies, cholesterol loading of murine VSMC increased their ABCA1 mRNA expression.31,32,67 Reconciling the apparent discrepancies between the results, which may have been caused by species differences, experimental conditions, etc, will require more investigation, but whatever the level of ABCA1 expression there is, it does not seem to be sufficient to prevent accumulation in vitro or in vivo when VSMC are exposed to elevated levels of cholesterol.

Second, though VSMC-derived foam cells exhibit impaired phagocytosis relative to macrophages, compared with contractile VSMC, they are considerably more active (>5fold).31 The cholesterol content of the more phagocytic cells would, therefore, be expected to contribute to VSMC-foam cell formation beyond the effects of hypercholesterolemia. In contrast to the data for phagocytosis, efferocytosis was not different in vitro between VSMC and cholesterol-loaded VSMC, suggesting that autophagic capacity may be submaximal in VSMC compared with macrophages.<sup>31</sup> As autophagy is an important factor providing intracellular cholesterol to the efflux pathway,45 its potential limitation might be a contributor to impaired VSMC-foam cell cholesterol efflux. Further aggravating the cellular cholesterol imbalance in VSMC-foam cells is that

apparently another homeostatic mechanism to promote efflux when cells are cholesterol-loaded, namely, the induction of LXR-regulated pathways, fails to become activated in vitro.<sup>73</sup>

If the current speculation that transitioned VSMC have negative contributions to atherosclerosis are true, then the value of restoring cholesterol efflux to intimal VSMC becomes clear. An interesting question arises: how do the efflux capacities of distinct foam cell populations differ from one another? The answer would have implications in designing therapeutic strategies to target all or a subset of foam cells in the plaque to maximally promote RCT.

#### **Reverse Cholesterol Transport**

Though HDL is thought to have many functions,74-76 overwhelmingly its ability to promote RCT is considered key to its atheroprotection. This has stimulated much research in enhancing RCT. Although this pathway has been actively studied for several years, mechanistic understanding of ABC family mediated lipid export and nascent HDL biogenesis remains incomplete, with basic pieces of the puzzle such as structural information of the ABC subfamily only just emerging.<sup>77</sup> Although ABCA1 preferentially lipidates small HDL particles, specifically apoA-I to form nascent HDL,78,79 ABCG1 stimulates net cholesterol efflux to larger HDL but not to lipid-poor apoA-I.80 Moreover, as alluded to above, ABCA1 trafficking between the cell surface and late endocytic vesicles is functionally important to stimulate cholesterol efflux out of endosomal/lysosomal compartments to lipid-free apoA-I,55,81,82 while ABCG1 is an intracellular sterol transporter that promotes cholesterol trafficking from the ER to the plasma membrane.<sup>59</sup> In turn, efflux to HDL involves passive diffusion of cholesterol as well as active cholesterol transfer, and ABCA1, ABCG1, as well as unrelated SR-B1 (scavenger receptor class B type 1), mediate lipid transfer to HDL.83-86 After cholesterol transfer to HDL particles, the next step in HDL biology is esterification of the acquired cholesterol by LCAT (lecithin:cholesterol acyltransferase) to form CE, giving rise to mature HDL. Remodeling of HDL particles can occur through the hydrolysis of HDL triglycerides and phospholipids, mediated by hepatic lipase and endothelial lipase, respectively.87 In humans (but not mice), CE in the HDL core can be transferred to triglyceride-rich lipoproteins by CETP (cholesteryl ester transfer protein) for elimination via hepatic clearance in the liver through the LDLR, or selectively taken up via SR-B1 acting as a hepatic receptor for CE on HDL. Therefore, RCT to the liver of cholesterol derived from peripheral cells in humans involves 2 routes; (1) direct (HDL-SR-B1) and (2) indirect (HDL-LDL/VLDL-liver LDLR). In the liver, the CE is hydrolyzed and the free cholesterol is either converted to bile acids or transported by ABCG5 and ABCG8 into the bile for excretion into the feces.

Three conceptual approaches to enhancing RCT have been proposed: (1) improve macrophage cholesterol efflux, (2) improve HDL functionality (ie, its capacity to accept or transport cholesterol), and (3) improve hepatic cholesterol uptake and biliary/intestinal excretion. 88 As research has continued, this third possibility has been informed by mounting evidence that several HDL-independent routes can promote RCT and that cholesterol removal from the body may not

require hepatobiliary cholesterol excretion.<sup>89</sup> Thus, the term RCT currently encompasses all potential routes of net cholesterol flux from peripheral tissues into the feces,<sup>90</sup> including artificial ones that have therapeutic potential. For example, non-HDL particles of 2-hydroxypropyl-β-CD (cyclodextrin) are artificial cholesterol acceptors and have been shown in vivo to mediate RCT and atheroprotection.<sup>91,92</sup>

Other modalities to increase RCT include liposomes, 93-95 the red blood cell compartment, which can act as a cholesterol sink to increase RCT,96 microparticle-mediated cholesterol efflux, 97 and synthetic nanoparticles and HDL mimetics that not only serve to package and deliver therapeutic drugs such as LXR agonists or statins to the arterial wall to stimulate cholesterol efflux but can also extract plaque cholesterol.98-100 There are also efforts to increase LCAT activity so that more free cholesterol can be esterified, increasing the amount loaded into HDL. 101 There is renewed interest in hepatic SR-B1 as a target, based on the work from Rader and colleagues showing loss of function SR-B1 mutations in people are associated with increased cardiovascular risk, despite elevated HDL-C. 102 This is consistent with mouse models in which deficiency of SR-B1 increased HDL-C but paradoxically increased atherosclerosis. 103 In these studies, SR-B1 deletion or loss of function impaired RCT, consistent with the growing body of evidence highlighting that HDL function and cholesterol flux are ultimately better determinants of atheroprotection than absolute HDL-C concentrations. However, it should be noted that another study found that rare mutations that disrupt SR-B1 function associates with HDL-C but not CAD risk. 104

In addition to the hepatobiliary route of cholesterol elimination, there is also transintestinal cholesterol efflux (TICE). While hepatobiliary cholesterol secretion involves the transfer of cholesterol from hepatocytes into the bile canaliculus, 105 in TICE cholesterol is transported directly from blood, through the enterocytes, into the lumen of the intestine. 106 These fecal cholesterol routes—hepatobiliary and TICE—are estimated to account for 65% and 35% of cholesterol elimination in humans, 106 respectively. The nuclear hormone receptors LXR and FXR (farnesoid X receptor) are important regulators of cholesterol excretion, by controlling the transcription and activity of numerous cholesterol transporters and bile synthesis enzymes. 105–107

Although cholesterol itself can be secreted into the bile for excretion from the body, synthesis, and excretion of bile acids comprise the major cholesterol catabolism pathway in mammals. Thus, LXR and FXR both represent potential therapeutic targets to stimulate TICE and biliary cholesterol secretion and promote RCT. 107,109 Because hepatic LXR activation also stimulates lipogenesis, leading to steatohepatitis, 110 devising a strategy to selectively activate nuclear receptors in the intestinal lumen to promote TICE without inducing hepatic lipogenesis may represent a targeted approach to circumvent this issue. In addition, miRNAs add an extra level of regulation to cholesterol metabolism by exerting post-transcriptional negative control of certain genes, including ABCB11 and ATP8B1, 111 suggesting anti-miRNA therapies.

#### **Quantification of RCT**

The controversy surrounding HDL-C as a reliable biomarker of HDL function, including the promotion of RCT, does not

contradict the view held by many that increasing RCT will contribute to reducing atherosclerosis and the risk of cardiovascular events. To Indeed, an independent inverse association between HDL cholesterol efflux capacity (CEC) and incident cardiovascular events has been shown both in the Dallas Heart Study and in the European Prospective Investigation of Cancer-Norfolk study. It addition, quantification of cholesterol mass efflux capacity in CAD and stroke cohorts derived from the Multi-Ethnic Study of Atherosclerosis indicate a protective role for HDL-mediated efflux in patients with CAD albeit not those with stroke. It Thus, considerable efforts have been made to develop measurements of RCT in vitro and in vivo, especially with an eye to test approaches to increase it, such as those suggested above.

In vitro, commonly used are assays of the first step in RCT, the efflux of cellular cholesterol. In this type of assay, cells are first incubated with radioactive [3H or 14C]-cholesterol or, alternatively, fluorescent BODIPY (boron-dipyrromethene)cholesterol to label intracellular cholesterol pools, after which transfer of the labeled cholesterol from the cells to an extracellular cholesterol acceptor, such as apoA-I or HDL, is measured over time. 117-119 One must consider several factors when designing a cholesterol efflux experiment, 120 for example, the exogenous cholesterol acceptor and label to be used, keeping in mind how this might affect net cholesterol flux given that efflux to α-HDL may be bidirectional, so that the correlation of BODIPY-cholesterol efflux and that of <sup>3</sup>H-cholesterol to preβ-HDL and α-HDL may differ. <sup>118</sup> A variation of these assays, originally developed by Rothblat, Rader, and colleagues,5 has been used to assess the CEC of HDL isolated from human subjects to determine its correlation between HDL CEC and cardiovascular risk. 114,115,121,122 A number of such studies (but not all<sup>123</sup>) have found an independent inverse association between HDL CEC and incident cardiovascular events, supporting HDL CEC as a metric of cardiovascular risk superior to HDL-C. Nevertheless, side-by-side comparisons of the radiolabeled and the fluorescently labeled cholesterol method is necessary to determine if this accounts for differences among studies and the correlation of HDL CEC with HDL-C.

Turning to assays in vivo, a simple assay developed by Rader et al has been used to quantify RCT in experimental mouse models. This consists of injecting macrophages loaded with radiolabeled cholesterol into the peritoneal cavity of mice, and measuring the appearance of the radiolabel into the plasma, liver, and feces over time. 124 The major limitation of this assay is that it does not consider the bidirectional movement of cholesterol in and out of macrophages, and thus one cannot draw conclusions about the net outward flux of cholesterol mass. To circumvent this, macrophage-specific RCT might be better quantified using techniques in which macrophages are trapped into the site of injection using semipermeable hollow fibers or Matrigel plugs, and these implants are removed so that cholesterol mass content may be determined at the end of the assay. 125,126 More recently, Cuchel et al 127 adapted the conventional RCT method to allow for quantification of RCT in humans. This method involves intravenous delivery of <sup>3</sup>H-cholesterol nanoparticles, followed by blood and sample collection to quantify tracer counts in plasma, non-HDL, and HDL fractions, as well as fecal fractions. This is an exciting advance in the field, providing a feasible approach to quantify RCT in vivo in humans. A combination of this methodology along with HDL CEC quantification and advanced modalities in imaging, such as intravascular ultrasonography, optical coherence tomography, and near-infrared spectroscopy to facilitate in situ plaque imaging may together provide a better assessment of whole-body RCT capacities in humans and allow for clinical testing of new drugs for the treatment of CAD.<sup>128</sup>

#### Selected Topics in Cholesterol Efflux, HDL Biology, and RCT

#### **Atherosclerosis Prevention and Regression**

A chronic inflammatory disease, atherosclerosis begins with the accumulation of apoB-containing lipoproteins and their cholesterol in the artery wall. In response to arterial lipoprotein/lipid buildup and retention, macrophages are recruited to the intima and take up the modified lipoproteins and their lipids by multiple processes, <sup>129</sup> leading to the formation of foam cells that secrete inflammatory mediators and promote the development of early atherosclerotic lesions. These lesions develop into disease-causing advanced plaques in the process commonly referred to as atherosclerosis progression. Once advanced atherosclerotic plaques are established, the process by which they undergo a reduction in one or more standard parameters (size, lipid content, foam cell content, and macrophage inflammation) is termed atherosclerosis regression. Macrophage RCT is the mechanism by which atherosclerotic plaques may rid themselves of cholesterol, and, as noted earlier, is still considered as an essential target to inhibit atherosclerosis progression and promote atherosclerosis regression.

Besides cholesterol efflux, macrophage RCT may also involve cholesterol removal from plaques by another mechanism, namely by the emigration of the macrophages themselves. Plaque foam cell population numbers are determined by cell recruitment, proliferation in situ, emigration, and cell death. 130 Historically, atherosclerosis progression studies have placed a major emphasis on understanding mechanisms of monocyte recruitment into the vascular wall and devising strategies to block their influx into plaques. 130 Recent studies, however, show that there are also factors that determine macrophage retention within and egress from plaques,130 and if these are manipulated appropriately, can lead to reductions in macrophage numbers and the cholesterol they contain, resulting in regression in murine models. One of the emigration factors is the CCR7 (C-C chemokine receptor type 7), 131 whose transcription is regulated in part by a sterol response element in its promoter. 132 When HDL levels were raised in ApoE-/mice, the SREBP pathway in plaque macrophages was activated, and macrophage emigration was stimulated. 132

Another study reported that raising HDL in *ApoE*<sup>-/-</sup> mice as a consequence of using an apoE-encoding adenovirus to reduce non-HDL hyperlipidemia decreased plaque macrophage content by 74% after 4 weeks of apoE complementation. This was attributed to a marked reduction in monocyte recruitment to plaques but not to CCR7-dependent egress of macrophages from plaques.<sup>133</sup> The role of CCR7 in some models of murine atherosclerosis regression was confirmed in a recent study, in

which it was shown that deficiency of LRP1 increased RCT and CCR7 expression in plaque macrophages, and promoted atherosclerosis regression, which was associated by the appearance of plaque macrophages in lymph nodes. <sup>134</sup> Thus, egress of macrophages and perhaps other leukocytes from plaques is likely a significant contributor to net RCT in certain, but not all, contexts (see below on Lymphatics and RCT). Whether foam cells of VSMC origin can also emigrate from plaques and the extent to which they may do so relative to classical macrophage foam cells remains to be determined.

#### **Lymphatics and RCT**

There is a growing interest in the role of lymphatics in RCT. Lymphatic capillaries have been localized in the adventitia of atherosclerotic plaques, where they play an important role in the drainage of local inflammatory cells and cytokines and protect against atherosclerosis development. 135 The lymphatic vasculature is also critical for the removal of cholesterol from macrophages in RCT, accounting for 50% of cholesterol delivery from cholesterol-loaded macrophages into the plasma compartment. 136 Moreover, lymphatic insufficiency in mice disrupts proper lipoprotein metabolism (eg, elevated cholesterol and triglyceride levels in VLDL and LDL fractions) and vascular homeostasis, leading to accelerated atherosclerosis. 137 These findings are in agreement with previous studies showing that interstitial fluid supports RCT; whereas plasma mainly contains α-HDL particles that are the predominant carriers of CE to hepatocytes, interstitial fluid provides a metabolic environment that drives the conversion of α-HDL to pre-β-HDL, the main acceptor of free cholesterol from peripheral tissues. 138

HDL particles can be partitioned into several subclasses according to the specific isolation or separation technique applied. By ultracentrifugation, 2 HDL subclasses can be obtained:  $HDL_2$  (1.063–1.125 g/mL) and  $HDL_3$  (1.125–1.21 g/mL). In turn, agarose gel electrophoresis separates HDL based on surface charge and shape into  $\alpha$ - or pre- $\beta$ -migrating particles ( $\alpha$ -HDL or pre- $\beta$ -HDL). Pre- $\beta$ -HDL primarily consists of poorly lipidated apoA-I and is the substrate for ABCA1 that transfers phospholipids and cholesterol to apoA-I to generate nascent discoidal HDL. In turn,  $\alpha$ -HDL represents mature HDL that arises from the esterification of free cholesterol into CE by LCAT, and  $\alpha$ -HDL can subsequently be further lipidated through the action of ABCG1 and SR-B1 (Figure).

Whether apoB-containing lipoproteins, which can also serve as cholesterol acceptors to facilitate RCT depending on the gradient, also enter peripheral tissues and drain into the lymph to regulate RCT remains to be investigated. In addition, more research is needed to understand how artery tertiary lymphoid organs form in the adventitia during atherosclerosis and to determine their role in regulating the immune response during atherosclerosis and how they may modulate RCT flux. For example, these lymphoid aggregates secrete chemokines that may promote foam cell retention, which may in turn increase plaque lipid burden. <sup>141</sup>

#### **Diabetes Mellitus**

As an example in which impairment in one or more components of RCT may underlie increased CVD risk, we will discuss

diabetes mellitus. Diabetes mellitus, both type 1 and type 2, represent significant global health issues, with CVD accounting for 65% of mortality in this population. 142 Additionally, the metabolic syndrome, a disorder associated with increased risk of developing type 2 diabetes mellitus, is unequivocally linked to increased risk for premature CVD and death. 143 Type 2 diabetes mellitus and the metabolic syndrome have a number of associated pathologies, including insulin resistance, obesity and high plasma triglycerides, and, relevant to RCT, low levels of HDL-C and apoA-I, reduced HDL particle (HDL-P) number and dysfunctional HDL-Ps. 144-150 Thus, there are a number of facets of RCT which likely contribute to heightened CVD risk in diabetic and metabolic syndrome patient populations.

One mechanism for the impaired HDL-P function may be related to the formation of advanced glycation endproducts (AGEs), which are nonenzymatic modifications of proteins that occur in vivo in patients with diabetes mellitus. Glycation of HDL and apoA-I is proposed to impair their functionality by reducing both their CEC and antioxidant capacity. <sup>18,151–156</sup> Additionally, in vitro, high glucose and AGE-modified proteins impair macrophage CEC by downregulation of the transporters ABCA1 and ABCG1, attributable to increased local production of reactive oxygen species. <sup>79,157–160</sup> Consistent with this, numerous murine and human studies report decreased expression of ABCA1 and ABCG1 in monocytes and macrophages isolated from diabetic mice and people, translating to decreased myeloid CEC. <sup>99,161–164</sup>

Mechanistically, reduced CEC transporter levels under diabetic conditions in vivo are mediated, in part, by the receptor for AGE (RAGE), 161,163 which would be expected to be stimulated by the AGE-production noted above. This was recently highlighted by Daffu et al163 who reported that incubation of murine macrophages or human THP-1 (human leukemic cell line) cells with the model glycated protein CML (carboxy methyl lysine)-AGE reduced Abcal and Abcgl mRNA and protein expression via its interaction with RAGE. Reductions in the expression levels of these receptors resulted in decreased cholesterol efflux to apoA-I and HDL. 163 Further, consistent with other studies, 165-169 it was found that diabetes mellitus enhanced both atherosclerosis progression and impaired regression and that global deletion of RAGE overcame these defects by restoration of ABCA1 and ABCG1, promoting macrophage CEC despite ongoing hyperglycemia. 163,170

Restoration of global and myeloid ABCA1/ABCG1 expression and improvements to CVD outcomes under diabetic conditions is likely to be multifaceted. In addition to being essential for the removal of cholesterol from plaque macrophages,<sup>28</sup> ABCA1 and ABCG1 regulate the proliferation of hematopoietic stem and progenitor cells to control the abundance of blood monocytes.<sup>27</sup> Given the link between myelopoiesis and CVD risk, 130,171 suppression of this process is likely to directly inhibit the progression of atherosclerotic lesions and promote lesion regression.<sup>166</sup> Diabetes mellitus can suppress hematopoietic precursor cell ABCA1 and ABCG1 levels, promoting myelopoiesis and atherosclerosis. 165 Furthermore, inhibition of miR-33, a negative regulator of cellular ABCA1 and ABCG1, suppresses leukocytosis and reduces plaque macrophage inflammation in diabetic mice. 165 Despite persisant hyperglycemia, suppression of miR-33 not

only restored essential cholesterol transporters and reduced myelopoiesis, but it also promoted inflammation resolution in established plaques. Additionally, unpublished work from the Fisher lab has found that raising apoA-I/HDL levels in diabetic mice, in the absence of glucose control, can restore atherosclerosis regression, in part, by overcoming defective CEC in hematopoietic stem cells (Barrett et al, In Revision). These complementary studies highlight the importance of effective CEC at both the level of the bone marrow and plaque under diabetic settings to reduce CVD morbidity and mortality risk.

As with nondiabetic populations, the relationship of HDL-C to effective RCT in diabetic patients with CVD risk remains to be conclusively determined. However, given that macrophage CEC to plasma from diabetic subjects is overwhelmingly reported to be reduced compared with healthy controls, 139,172-175 and the inverse relationship between glucose tolerance and plasma CEC, 115,176 it is tempting to speculate that either restoring or enhancing in vivo RCT capacity within this population would reduce the incidence of CVDlinked disorders.

#### Functional Properties of HDL in Cholesterol Efflux, RCT, and Beyond

Factors to consider about the functionality of HDL include its pleiotropic actions besides cholesterol efflux. The bases for these actions likely involve the heterogeneity of HDL particles. For example, independent of its ability to mediate RCT by serving as a cholesterol acceptor, HDL is also known to exert potent antioxidant and anti-inflammatory effects that can improve RCT, retard plaque progression, and promote plaque regression.<sup>75,177</sup> Indeed, overexpression of an HDL-associated protein that confers antioxidant properties to HDL, paraoxonase 1, improves the efflux capacity of HDL, and drives RCT in mice. 178 HDL exists as subpopulations, classified based on their physicochemical properties: density (HDL<sub>2</sub> and HDL<sub>3</sub>), shape (discoidal and spherical), protein (apoA-I, A-II, or both), surface charge, and size. 139 The bulk of RCT is linked to apoA-I, which cycles between lipid-poor (pre- $\beta$ -HDL) and -rich ( $\alpha$ -HDL) forms of HDL, a remodeling event that, as noted earlier, can occur in the interstitial fluid of tissues to generate pre-β-HDL. This process is essential to RCT given that just 5% of plasma apoA-I exists as pre-β-HDL, the principal acceptors of cholesterol from peripheral cells. 138,179,180 Proteomic analyses reveal that the composition of HDL is more complex than anticipated, containing ≈200 diverse proteins distributed among various HDL subclasses. In addition to protein and lipid cargo, HDL can transport functional noncoding RNAs, such as miRNAs, and this pool of lipoprotein-associated RNA can be altered in disease. 181,182 It is now appreciated that how specific HDL functions (in CEC/RCT, thrombosis, inflammation, etc) are related to HDL compositional heterogeneity in humans and how HDL subspecies may be altered during CAD could lead to the identification of new diagnostic tools and therapies. 139,183-185

#### **Concluding Remarks**

The quest for HDL-raising therapies has been long-standing in the fields of lipoprotein metabolism and CVD, as reflected in the past by physicians routinely prescribing drugs to boost HDL-C in patients. These therapies are now thought to be ineffective in reducing CVD risk. 186 In addition, several clinical studies failed to show that raising HDL-C levels (eg, by niacin<sup>187,188</sup> or CETP inhibition<sup>189</sup>) improves CVD outcomes, and Mendelian randomization studies also find that HDL-C levels are not predictive of CVD events.<sup>183</sup> These and other studies highlight that while we have observed numerous successes in the development of multiple LDL-cholesterol lowering therapies that have translated into beneficial clinical outcomes, comparable advances in RCT-enhancing strategies through raising HDL-C are lacking. An example of the need for such enhancement independent of HDL-C may be found in the data from the CETP inhibitor trials. In particular, it may be more than a coincidence that the failure of torcetrapib to lower CVD events despite raising HDL-C by ≈72% 190 was also associated with its failure to promote whole-body RCT in a fecal sterol excretion assay.191

It should be noted that in none of the studies mentioned above and in many similar ones has HDL function been ascertained, leaving open the possibility that HDL function is the key attribute for CVD risk reduction. 113 HDL function as a clinically important factor has found traction not only in the aforementioned CEC studies, but also finds some support from some but not all (eg, Nicholls et al192) infusion studies of recombinant HDL and HDL-like particles. Notably, however, all of the infusion studies to date are of limited significance, as they have been either too short to assess effects on CVD outcomes, very small in subject number, or both. For example, in one small study, the intravenous infusion of a single dose of reconstituted HDL led to acute changes in plaques in the superficial femoral artery, with a reduction in lipid content, macrophage size, and measures of inflammation, but there were only 20 subjects. 193 In a larger study of subjects post-acute coronary syndromes (47 subjects completed the protocol), 5 weekly injections of a recombinant HDL-like particle (designated ETC-216) containing ApoA- $I_{\rm milano}$ -phospholipid complexes  $^{194}$  resulted in a 4.2%decrease from baseline in coronary atheroma volume as measured by intravascular ultrasound.

A similar study was conducted with a formulation of wildtype ApoA-I (designated CSL111). 195 The results were similar to the ETC-216 trial, in that plaque volume was decreased, but to a lesser extent (3.4%), perhaps because of a shorter course of treatment (4 weeks) or other differences between the studies. Again, there are no CVD outcome data in either trial. There is great interest, therefore, in the AEGIS II trial (ApoA-I Event Reducing in Ischemic Syndromes II), in which apoA-I in a proprietary formulation of lipids to simulate HDL particles (CSL112), is being administered to subjects with the acute coronary syndrome. With an estimated enrollment of 17400, participants will be randomized to receive either CSL112 or a placebo, administered through intravenous infusion once a week for 4 consecutive weeks. The primary end point is the first occurrence of a major adverse cardiovascular event, cardiovascular death, myocardial infarction, or stroke within 90 days, and the expected completion date is 2022.<sup>187</sup>

In spite of the controversies, on-balance we think that raising levels of functional HDL in those at risk for CVD events may yet represent a viable therapy to suppress atherosclerosis progression and promote atherosclerosis regression.

This belief is based on the established biological effects of functional HDL that we have summarized, as well as the encouragement from the clinical studies, (114,193–195) although at present they fall short as definitive trials, especially with regard to the relationship between raising levels of functional HDL and MACE. This raises the parallel need for more trials of the type that AEGIS II represents, as well as mechanistic studies to further understand the factors that regulate HDL's impact on CVD independent of the plasma concentration of HDL-C.

#### **Sources of Funding**

This work was supported by funding from the Canadian Institutes for Health Research (PJT-391187 and Canada Research Chair to M. Ouimet), the Heart and Stroke Foundation of Canada (M. Ouimet), the American Heart Association (18CDA34110203AHA; T.J. Barrett), the National Institutes of Health (DK095684, HL084312, HL129433, HL092969, HL122728, HL117226, and HL131481; E.A. Fisher), and the Department of Defense (W81XWH-15-1-0374, W81XWH-16-1-0255; E.A. Fisher).

#### **Disclosures**

None.

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