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*J Immunol* 2013; 191:4326-4336; Prepublished online 18 September 2013; doi: 10.4049/jimmunol.1300990

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http://www.jimmunol.org/content/suppl/2013/09/18/jimmunol.1300990.DC1

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Conjugated Linoleic Acid Targets β2 Integrin Expression To Suppress Monocyte Adhesion

Monica de Gaetano,1 Eugene Dempsey,1 Simone Marcone, William G. James, and Orina Belton

Chronic recruitment of monocytes and their subsequent migration through the activated endothelium contribute to atherosclerotic plaque development. Integrin-mediated leukocyte adhesion is central to this process. Conjugated linoleic acid (CLA) has the unique property of inducing regression of pre-established murine atherosclerosis via modulation of monocyte/macrophage function. Understanding the mechanisms through which CLA mediates its atheroprotective effect may help to identify novel pathways that limit or reverse atherosclerosis. In this study, we identified a novel mechanism through which CLA alters monocyte function. We show that CLA inhibits human peripheral blood monocyte cell adhesion to activated endothelial cells via loss of CD18 expression, the β2 chain of LFA-1 and Mac-1 integrins. In addition, using a static-adhesion assay, we provide evidence that CLA prevents monocytes from binding to ICAM-1 and subsequently reduces the capacity of these cells to polarize. CXCL12-CXCR4 interactions induce a conformational change in β2 integrins, facilitating leukocyte adhesion. In this study, we demonstrate that CLA inhibits CXCR4 expression, resulting in a failure of monocytes to directionally migrate toward CXCL12. Finally, using intravital microscopy, we show that, during CLA-induced regression of pre-established atherosclerosis in ApoE−/− mice, there is reduced leukocyte adhesion and decreased CD18 expression on Gr1+/CD115+ proinflammatory monocytes. In summary, the data presented describe a novel functional role for CLA in the regulation of monocyte adhesion, polarization, and migration. The Journal of Immunology, 2013, 191: 4326–4336.

Atherosclerosis, the underlying cause of heart disease and stroke is a complex progressive disease with multiple genetic and environmental contributions. In addition to the role of lipids, atherosclerosis has many features of an inflammatory disease, including infiltration of monocytes to the vessel wall and a proliferative response of smooth muscle cells, for example. Early events in the pathogenesis of the disease involve disturbed fluid shear stress, particularly at branch points or bifurcations, such as the aortic arch and iliac arteries, resulting in dysfunction of the endothelium on blood vessels (1–3) and, subsequently, increased vascular permeability to macromolecules, including cholesterol containing low-density lipoprotein (LDL). Subendothelial deposits of LDL become oxidized to produce minimally oxidized LDL, which induces local inflammation and subsequent infiltration of immune cells. Although many cell types, including dendritic cells, T cells, endothelial cell, and smooth muscle cells (4, 5), are documented to play a role in atherogenesis, the chronic recruitment of monocytes to the developing plaque and their differentiation to macrophages are central elements in the continual progression of the disease (6, 7).

The recruitment of monocytes to inflammatory sites is a highly orchestrated process in which integrins play a central role. Integrins are a large family of multifunctional heterodimeric transmembrane proteins consisting of an α- and β-chain that mediate rolling and adhesion of leukocytes. Integrins on circulating leukocytes are typically present in low-affinity states; however, a mechanism initiated by chemokines, such as IL-8 and CXCL12 (8, 9), termed “inside out” signaling, induces their conformational change to intermediate or high-affinity states that results in increased binding affinity for their respective ligands (10). The β2 integrins LFA-1 and Mac-1 share a common β-chain (CD18), which is non-covalently linked with a unique α-chain: CD11a (αL) or CD11b (αM), respectively. Both β2 integrins bind ICAM-1 (11), which is upregulated on endothelial cells in the presence of proinflammatory cytokines, such as TNF-α. Sumagin et al. (12) recently demonstrated the differential roles of LFA-1 and Mac-1 on monocyte crawling and showed that LFA-1 is used by monocytes to crawl on unactivated endothelial cells; however, during TNF-α-induced inflammation, Mac-1 becomes the more important integrin for attachment and crawling.

Most of the cellular and molecular mechanisms involved in the development of atherosclerotic lesions have been identified; however, there has been no defined pathway that would explain how this process could be reversed or even if such pathways exist. Conjugated linoleic acid (CLA) is a generic term denoting a group of naturally occurring isomers of linoleic acid (18:2, n6), which differ in the position and geometry of their double bonds. CLA has been investigated as a potential prophylactic intervention for multiple inflammatory diseases, including obesity, hyperinsulinemia, and hypertension (13, 14). Additionally, CLA was identified to have antiatherogenic effects in a number of animal models of atherosclerosis (15–17). The most potent immuno-modulatory CLA isomers are cis-9,trans-11 CLA and trans-10,
mented ApoE protein–coupled receptor, leading to a failure of monocytes to cells. We also show that this is mediated by inhibition of CLA, either individually or as an 80:20 ratio blend, on monocyte supplementation (20). In this study, we aimed to define the mechanisms through which CLA alters monocyte function and confers an atheroprotective phenotype. To this end, we examined the effect of the two most abundant CLA isomers, cis-9,trans-11 CLA and trans-10,cis-12 CLA, either individually or as an 80:20 ratio blend, on monocyte adhesion. We demonstrate that CLA inhibits human peripheral blood monocyte cell (HPBMC) adhesion to activated endothelial cells. We also show that this is mediated by inhibition of β2 integrins via a PPARγ-dependent mechanism. Additionally, we demonstrate that CLA also inhibits the expression of CXCR4, a G protein–coupled receptor, leading to a failure of monocytes to directionally migrate toward the chemokine CXCL12. Finally, using intravital microscopy (IVM), we show that CLA-supplemented ApoE−/− mice have a reduction in the attachment of leukocytes in areas of vascular inflammation in vivo. The data presented in this article describe a novel functional role for CLA in the regulation of monocyte adhesion, polarization, and migration.

Materials and Methods

Animals and diets

Homoygous ApoE-deficient mice (002052) (C57BL/6J-ApoE<sup>−/−</sup>) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were conducted in conformity with institutional guidelines and in compliance with international laws. An 80:20 cis-9,trans-11 CLA:trans-10,cis-12 CLA blend was supplied by Stepian Lipid Nutrition (Wormerveer, Holland) and incorporated into 1% cholesterol diet (CD; Special Dietary Services, Essex, U.K.). ApoE−/− mice were randomized at 40 d to receive CD for 12 wk or CD for 12 wk during which the final 4 wk was supplemented with 1% CLA blend. This diet protocol was shown to promote regression of pre-established atherosclerotic lesions in this model (20). ApoE−/− mice maintained on a normal cholesterol chow diet (ND) for the 12-wk period were also examined.

Isolation of HPBMCs

Whole blood from healthy volunteers was drawn into heparin-coated vacutainers (BD - UK/Ireland). All volunteers were nonsmoking, aged 25–30 y, and free from medication for ≥10 d. Platelet-rich plasma was isolated by centrifugation (190 × g for 15 min), diluted 1:3 with PBS before addition to Lymphoprep (A/S SHIELD PC, Oslo, Norway), and centrifuged at 450 × g for 30 min. Buffy coats were recovered using a Pasteur pipette, washed twice with PBS, and resuspended in 10 ml serum-free medium (SFM) M-199 (Thermo Scientific), supplemented with t-glutamine (6.8 mM), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and 10 ng/ml polymyxin B sulfate (Sigma-Aldrich, Dublin, Ireland). Monocytes were purified by plastic adherence in 2 h. Attached monocytes were washed and gently scraped from the surface before use in subsequent assays.

HPBMC culture and treatments

Prior to specific assays, monocytes were incubated at 37°C in SFM and treated with 10 μM cis-9,trans-11:trans-10,cis-12 (80:20) CLA blend, oleic acid (OA; used as fatty acid control), the PPARγ agonist troglitazone (TROG; 5 μM), or DMSO for 18 h. CLA and OA were purchased from Cayman Chemicals (Ann Arbor, MI); TROG was from Sigma-Aldrich.

Static-adhesion assay of monocytes to immobilized human aortic endothelial cells

Human aortic endothelial cells HAECS (Cascade Biologies, Invitrogen, Carlsbad, CA) were grown in endothelial cell culture media MV plus growth supplements from Promo Cell (Heidelberg, Germany), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. For experimental analysis, HAECS were incubated in growth media with 1% FCS, 0.4% endothelial cell growth supplements, 90 μg/ml heparin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidified atmosphere of 5% CO2 at 37°C.

For static-adhesion assays, 4 × 10⁴ HAECS were seeded in a 96-well plate for 24 h, followed by stimulation with TNF-α (10 ng/ml) for 6 h, and washing in media. Peripheral blood monocytes were labeled with fluorescein using 1 mg/ml BCECF-AM (Calbiochem), as per the manufacturer’s instructions. Subsequently 1 × 10⁵ labeled monocytes were added to activated HAECS and coincubated for 30 min at 37°C. Cells were washed three times with media, and fluorescence was measured in a Spectramax M2 (Molecular Devices, Sunnyvale, CA) plate reader with 485-nm excitation and 585-nm emission wavelength, before and after washing. A fluorescent intensity unit/cell was derived by dividing the total fluorescent signal before washing by the number of cells seeded. The final number of adherent cells was obtained by multiplying this unit by the total fluorescent signal after washing.

RNA extraction and gene-expression analysis

For gene-expression experiments, monocytes were washed twice with ice-cold PBS prior to addition of 200 μl Rlter buffer (QIAGEN, Manchester, U.K.). Total RNA was isolated from cell lysates using the RNeasy kit (QIAGEN), as per the manufacturer’s instructions. Reverse transcription was carried out on 1 μl total RNA using Superscript II Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. Relative gene expression quantification by real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, U.K.). β1 and β2 integrins, PSGL-1, and CXCR4 expression was examined using specific TaqMan assays (Applied Biosystems) and normalized to 18S rRNA.

Western blot and protein-expression analysis

Whole-cell lysates were prepared from monocytes in lysis buffer (0.2% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl [pH 7.2]) and then cleared by centrifugation (10,000 × g for 10 min at 4°C). Lysates were separated using 4–20% SDS-polyacrylamide gel and transferred to nitrocellulose (Hybond-C Extra; Amersham Biosciences, Buckinghamshire, U.K.). Membranes were blocked with 3% BSA in TBST (20 mm Tris [pH 7.6], 150 mM NaCl, 0.05% Tween 20) at room temperature prior to overnight incubation with primary Abs at 4°C with gentle shaking. Detection was performed using an HRP-conjugated secondary Ab (1 h at room temperature) and ECL detection system (Amersham Biosciences). mAb against β-actin (1:1000) and polyclonal Abs against CD11a (1:500) and CD11b (1:150) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). mAb against CD18 (RE72B; 1:3000) was a kind gift from Prof. Carl Gahmberg’s laboratories (Division of Biochemistry, Department of Biosciences, University of Helsinki, Helsinki, Finland).

Flow cytometry analysis

To block unspecific binding, monocytes were incubated in 1% BSA in PBS for 15 min prior to Ab staining. FITC-CD14 (clone UCHM-1; Sigma-Aldrich), PE-LFA-1 (BioLegend, London, U.K.), allopurinol–Mac-1 (BioLegend), and AF700-CD18 (EXBIO) Abs were used to label monocytes. All Ab incubations were for 30 min at room temperature. Cells were washed and fixed in 2% paraformaldehyde. Forward and side scatter gates were established to exclude cell debris from the analysis. The mean fluorescence intensity of 1 × 10⁵ cells was analyzed in each sample. Autofluorescence signals generated by unlabeled cells were used as neg-
ative controls in each experiment. Flow cytometric analysis was performed on an Accuri C6 instrument and analyzed with CFlow Software (Accuri, Ann Arbor, MI).

**Static-adhesion assay of monocytes to immobilized ICAM-1**

Ninety-six–well glass-bottom imaging plates (Zell-Kontakt, Nörten-Hardenberg, Germany) were coated overnight with recombinant human ICAM-1 protein (5 μg/ml; R&D Systems), washed with Ca²⁺ and Mg²⁺–free PBS (Life Technologies, Invitrogen), and blocked with 2% BSA (Sigma-Aldrich) for 2 h at 37°C. Following washes in Ca²⁺ and Mg²⁺–free PBS, 1 × 10⁵ fluorescein-labeled (using 1 mg/ml BCEFC-AM; Calbiochem) HPBMCs were treated with EGTA (1 mM) and MgCl₂ (5 mM) to induce the active conformation of surface integrins. Cells were rapidly added to ICAM-1–coated wells and incubated for 30 min at 37°C. Poly-L-lysine (0.01%; Sigma-Aldrich) coated wells were used as a positive control. Changes in adhesion were calculated as previously described.

**Immunocytochemistry**

To visualize adhesion and polarization of monocytes, 1 × 10⁵ cells were cultured and treated in a 96-well imaging glass plate (Zell-Kontakt). Adherent monocytes (activated with either EGTA/MgCl₂ or CXCL12) were fixed in 4% formaldehyde, permeabilized with Triton X-100 (Sigma-Aldrich), and labeled with Alexa-Fluor 568–phalloidin (Invitrogen) to stain F-actin and with DAPI (Sigma-Aldrich) to visualize nucleic acids. Images of stained monocytes were acquired using a Zeiss AxioVert 200M Inverted Fluorescent Microscope, and images were captured using an AxioCam HR (Zeiss) camera. CCD Basic resolution; 10×(air)/0.25 and 63×(oil)/1.40 lenses were used at room temperature. When needed, immersion oil, type DF (Cargille Laboratories) was used.

**CXCL12-induced crawling assay**

For transmission light microscopy acquisition of live cells, freshly isolated peripheral blood monocytes were treated as described above and incubated at 37°C for 18 h in a 96-well uncoated bare plastic plate (Greiner Bio-One, Cruinn Diagnostics, Dublin, Ireland). CXCL12 was added to one side of the well using either 1 ng/ml for THP-1 (in 1 ml) or 100 ng/ml for primary human monocytes (in 10 μl) to induce directional crawling. Immediately after addition of CXCL12, images were captured from the opposite side of the well at regular intervals over a period of 5–10 min, using a NIKON-TMS microscope, 20×(air)/0.4 lens, at room temperature. Pictures were captured using a 3-CCD Color Video Camera (JVC), KY-F55B. Matrix Intellicam acquisition software was used. Image sequences were analyzed, with x and y coordinates being assigned to individual cells; cell tracks were subsequently visualized using rosette plots.

**FIGURE 1.** CLA inhibits HPBMC adhesion to activated endothelial cells. (A) HAECs were activated following stimulation with 10 ng/ml TNF-α for 6 h. HPBMCs were treated with 10 μM *cis*-9,*trans*-11 CLA (c9, t11), *trans*-10,*cis*-12 CLA (t10,c12), CLA blend, OA, 5 μM TROG, or DMSO vehicle control (VC) for 18 h. Data are mean ± SEM. (B) MTT assay showing viability of HPBMCs following treatment with a dose range of c9,t11 and t10,c12 CLA. A total of 10 μM of each isomer was used for all subsequent experimental analyses. *p < 0.05, **p < 0.01 versus TNF-α–activated DMSO vehicle, $$$p < 0.001 versus resting DMSO control.
Leukocyte recruitment within the femoral artery was imaged by IVM. Animals from each diet regimen were randomly allocated into two treatment groups, a control saline injection or an acute dose of TNF-α (1 µg), as previously described (26). Briefly, 200 µl saline or TNF-α (1 µg in 200 µl saline) was administered i.p. 4 h prior to IVM imaging. Animals were anesthetized by i.p. injection of a mixture of xylazine (10 mg/kg) and ketamine hydrochloride (150 mg/kg) (C&M Vetlink, Limerick, Ireland). A catheter was inserted into the right jugular vein to administer anesthetic and fluorescent dye. To visualize leukocytes within the vasculature, 50 µl Rhodamine 6G (Sigma-Aldrich) (0.05% in saline) was administered i.v. prior to surgical exposure of the femoral artery (27). Animals were placed in a supine position on a temperature-controlled heating mat to maintain core temperature at 37°C.

Skin was incised along the interior surface of the thigh, and the femoral artery on each leg was exposed in turn for imaging. PBS superfusion was applied to the tissue. Fluorescent emission from Rhodamine 6G was visualized by epifluorescent microscopy via a 546/12-nm excitation filter and a 575–640-nm emission filter (Filter#20; Carl Zeiss U.K.). The femoral artery was imaged using a Carl Zeiss Axioskop 2 F-s-mot intravital microscope with a 20× water-immersion objective lens (20×/0.5 NA; Carl Zeiss U.K.). A Baxall CDX9742 monochrome camera (Carl Zeiss U.K.) collected the emitted light, which was displayed on a video monitor and recorded on a hard-drive recorder (JVC SR-DVM70) for playback analysis. The femoral arteries (150–190 µm in diameter) of both legs were imaged in the direction from the knee toward the body, with ~10–12 randomly selected fields-of-view recorded per artery. The CCD camera was rotated to position the artery in a horizontal orientation on the monitor to produce a standardized vessel length of 278 µm for each recording. Parameters analyzed in each recording included the leukocyte rolling flux (rolling cells/min), leukocyte rolling velocity (µm/sec), and the number of leukocytes adherent to the artery endothelium (adherent cells/vessel length). Leukocytes were deemed to be stably adhered if they remained stationary for ≥30 s. Because of the relatively large diameter of the femoral artery, depth of imaging was restricted to the outer endothelial surface.

Statistical analysis

Results

CLA inhibits human monocyte adhesion to endothelial cells

We demonstrated previously that dietary administration of 1% CLA blend (80:20, cis-9,trans-11:trans-10,cis-12) induces regression of pre-established atherosclerosis in the ApoE−/− mouse (20) and that modulation of monocyte function plays a central role in CLA-induced regression (21, 28). To examine this further, we investigated the effect of CLA on monocyte adhesion to activated endothelial cells. MTT assays were used to determine the optimum concentration of CLA isomers for all assays; 10 µM of cis-9, trans-11 CLA and trans-10,cis-12 CLA was the lowest dose of each isomer that did not affect cell viability (Fig. 1) but is known to have biological effects (21); hence, this concentration was used for all subsequent experimental analyses. Activation of HAECs with TNF-α induced a significant increase in the number of adherent monocytes (Fig. 1). Monocytes that were pretreated with cis-9,trans-11 CLA and 80:20 cis-9,trans-11:trans-10,cis-12 CLA

MTT assay

For this colorimetric assay, the following dose response was applied for each CLA isomer (cis-9,trans-11 and trans-10,cis-12): 0, 1, 10, 25, or 50 µM. After 18 h of treatment, 5 mg/ml MTT (Sigma Aldrich) solution was added and incubated at 37°C for 3.5 h. After cell lysing with DMSO, absorbance was read spectrophotometrically at 570 nm.

Results are expressed as mean ± SEM or fold change relative to vehicle control. All data are from a minimum of three independent experiments (n = 3). Statistical comparisons between controls and treated groups were made using the unpaired Student t test, assuming unequal variance. A p value <0.05 was considered significant.

FIGURE 2. Effect of CLA on β1, β2 integrins and PSGL-1 mRNA expression in HPBMCs. Peripheral blood monocytes were treated as previously described and analyzed for mRNA expression of the common β subunit (CD18) of the β2 integrin receptor (A), LFA-1 α-chain (CD11a) (B), Mac-1 α-chain (CD11b) (C), β-chain (CD29) (D) and α-chain (CD49d) of the αMβ2 integrin (E), and PSGL-1 (F). cis-9,trans-11 CLA and the CLA blend inhibit CD18 expression; trans-10,cis-12 CLA has no effect on CD18 but reduces PSGL-1 expression; and TROG inhibits β1, β2, and PSGL-1. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle control (VC).
blend had an attenuated ability to bind to activated endothelium, displaying a 63% ($p < 0.01$) and 68% ($p < 0.01$) decrease in the number of adherent cells, respectively. Treatment with trans-10, cis-12 CLA also had an inhibitory effect on adhesion, displaying a modest, but significant, 38% decrease in adhesion ($p < 0.05$). In contrast, OA was used as a fatty acid control and had no significant effect on monocyte adhesion. PPAR$\gamma$ is a known target of CLA (24); TROG, a PPAR$\gamma$ agonist, mimicked the CLA treatments by inhibiting adhesion by 58% ($p < 0.05$), suggesting that CLA inhibition of monocyte adhesion may be mediated via a PPAR$\gamma$-dependent mechanism.

**CLA inhibits the expression of adhesion molecules in peripheral blood monocytes**

To identify a possible mechanism by which CLA inhibits monocyte adhesion to activated endothelium, we analyzed the mRNA expression of a number of integrin and selectin receptors, including the $\beta_1$ integrin, VLA-4 ($\alpha_4\beta_1$), $\beta_2$ integrins (LFA-1 [$\alpha_L\beta_2$] and Mac-1 [$\alpha_M\beta_2$]), and P-selectin glycoprotein ligand-1 (PSGL-1), in CLA-treated human primary monocytes. Primary monocytes treated with either cis-9, trans-11 CLA or CLA blend experienced a significant decrease in the expression of CD18 by 1.92-fold ($p < 0.05$) and 1.53-fold ($p < 0.001$), respectively (Fig. 2A), whereas neither treatment significantly influenced the expression of CD11a, CD11b, CD29, or CD49d (Fig. 2B–E). Although the trans-10,cis-12 CLA isomer reduced the expression of CD18 by 1.47-fold, this was not significant. Indeed, with the exception of PSGL-1, which showed a significant 1.37-fold decrease ($p < 0.05$), trans-10,cis-12 CLA had no significant effect on any of the other mRNA transcripts examined. In contrast to CLA, the fatty acid control OA had no effect on the expression on $\beta_2$ integrin subunits and, in fact, increased the expression of CD49d and PSGL-1 by 1.3-fold ($p < 0.05$) and 1.5-fold ($p < 0.05$), respectively. TROG had a modest effect on most of the transcripts examined; however, it was a potent suppressor of CD18, resulting in a 4.35-fold ($p < 0.001$) decrease in expression.

Using Western blot analysis, we confirmed that the CLA-induced decrease in CD18 mRNA expression translated to a reduction in CD18 protein expression in CLA-treated HPBMCs. Relative to DMSO controls, CD18 protein expression was reduced by 44% in HPBMCs treated with cis-9,trans-11 CLA ($p < 0.05$), by 59% in HPBMCs treated with trans-10,cis-12 CLA ($p < 0.01$), and by 35% in HPBMCs treated with 80:20 CLA blend ($p < 0.05$) (Fig. 3A, 3B). Furthermore, there were no changes in expression of either of the $\alpha$-chains, CD11a or CD11b, following CLA treatment. In keeping with the mRNA-expression analysis, OA had no significant effect on the expression of CD18, whereas TROG potently inhibited CD18 protein expression by 90% ($p < 0.01$).

**FIGURE 3.** Effect of CLA on $\beta_2$ integrin protein expression in HPBMCs. HPBMCs were treated as described previously. (A) Western blot analysis of CD18, CD11a, and CD11b protein expression in activated monocytes treated with CLA. $\beta$-actin was used as a loading control. (B) Densitometric analysis of Western blot for CD18 (top panel), CD11a (middle panel), and CD11b (bottom panel). (C) Flow cytometry analysis showing percentage of CD14$^+$ cells expressing CD18, CD11a, or CD11b. (D) MFI percentage relative to DMSO of surface CD18 expression. Flow cytometry analysis showed that both cis-9, trans-11 CLA and the CLA blend decrease surface expression of all the subunits forming either LFA-1 or Mac-1 proteins. Data for (B) and (C) are expressed as percentage of protein expression over control. *$p < 0.05$, **$p < 0.01$ versus vehicle control (VC).
relative to controls. To confirm a PPARγ-dependent mechanism for the CLA-mediated regulation of CD18 expression, monocytes were treated with 10 μM of the PPARγ antagonist GW9662 for 2 h prior to the addition of CLA for 18 h (28). In the presence of the PPARγ antagonist, the effect of CLA on CD18 gene expression is lost, confirming a PPARγ-dependent mechanism for the regulation of CD18 gene expression (Supplemental Fig. 1).

Because integrins are integral membrane proteins, in the next series of experiments we used flow cytometry to identify whether there was a loss of cell surface integrin expression in CLA-treated monocytes. By gating on CD14+ monocytes, we assessed the expression of the β2 integrin subunits. Because of the potential for interindividual variability between three human donors, we calculated both the percentage median fluorescence intensity (MFI) relative to DMSO and the percentage of monocytes expressing the β2 integrin subunits. Compared with controls, cis-9,trans-11 CLA and CLA blend decreased the number of monocytes expressing the β-chain by 10% (p < 0.05) and 20% (p < 0.01), respectively (Fig. 3C). This was confirmed by analysis of the MFI percentage relative to DMSO control of cellular AF700-CD18 (Fig. 3D).

Additionally, the cis-9,trans-11 isomer decreased expression of both CD11a (by 13%, p < 0.05) and CD11b (by 8%, p < 0.05). Furthermore, the 80:20 blend followed the same trend as did cis-9, trans-11 CLA, but to a greater degree; CD11a-expressing cells were decreased by 30% (p < 0.01), whereas CD11b-expressing cells were decreased by 27% (p < 0.01). Neither the trans-10,cis-12 isomer nor OA had any effect on CD18, CD11a, or CD11b expression.

In contrast to the mRNA-quantification and Western blot data, TROG induced a significant increase in the number of expression relative to controls. To confirm a PPARγ-dependent mechanism for the CLA-mediated regulation of CD18 expression, monocytes were treated with 10 μM of the PPARγ antagonist GW9662 for 2 h prior to the addition of CLA for 18 h (28). In the presence of the PPARγ antagonist, the effect of CLA on CD18 gene expression is lost, confirming a PPARγ-dependent mechanism for the regulation of CD18 gene expression (Supplemental Fig. 1).

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Nonetheless, when these data are taken together, they suggest that CLA-mediated inhibition of monocyte adhesion to HAECs is due to a loss of expression of CD18, the β2 chain of both LFA-1 or Mac-1 integrins, and is potentially mediated via a PPARγ-independent mechanism.

**CLA-treated peripheral blood monocytes fail to adhere to ICAM-1 or develop a migratory phenotype**

The major ligand for β2 integrins is ICAM-1; therefore, we next aimed to identify whether the CLA-induced reduction in surface CD18 prevented monocytes from binding to ICAM-1. In a static-adhesion assay using plate-bound rICAM-1, we examined the effect of CLA isomers on the binding of monocytes using Mg++ and EGTA to induce the active conformation of integrins and showed that ICAM-1 significantly increases adhesion, only upon monocyte activation (Supplemental Fig. 2). This strategy was used, because activation of integrins with this method is dependent on the surface expression of the integrins (29). In further experiments, we showed that cis-9,trans-11 CLA significantly inhibited the binding of monocytes to ICAM-1 by 82% (p < 0.001). Furthermore, the 80:20 CLA blend was equally potent, with an 89% (p < 0.001) reduction in the number of adherent cells. In contrast, trans-10,cis-12 CLA had the opposite effect and increased adhesion by 28% (p < 0.05) (Fig. 4A, 4B). A 61% increase in adhesion (p < 0.05) was observed in OA-treated cells, whereas a 75% increase (p < 0.05) was seen in TROG-treated cells.

Leukocytes binding to ICAM-1 develop a polarized promigratory phenotype with an actin-rich leading edge and a trailing uropod (30). To examine whether CLA treatment could also prevent polarization of monocytes, we pretreated monocytes with CLA as before and incubated them on ICAM-1–coated plates.
Cells were subsequently stained for F-actin and analyzed. Monocytes treated with either cis-9,trans-11 CLA or the CLA blend maintained a rounded phenotype, whereas all other treatment conditions allowed the monocytes to polarize (Fig. 4C). Interestingly,

**FIGURE 5.** CLA prevents ICAM-1–induced polarization on CXCL12-activated HPBMCs. (A) Following activation of HPBMCs for 1, 5, or 10 min with 100 ng/ml CXCL12, cells were fixed and stained. Fluorescent microscopy images of monocytes labeled with DAPI (blue) and Alexa Fluor 647–phalloidin (red). Images are representative of three independent experiments. Scale bars, 10 mm. (B) RT-PCR analysis of mRNA expression of CXCR4 in HPBMCs. (C) Cell tracking of CXCL12-induced crawling in THP-1 monocytes. Plots show individual tracks from 15 cells normalized to their starting position. cis-9, trans-11 CLA–treated, CLA blend–treated, and unstimulated vehicle control (VC)-treated cells do not spread on ICAM-1–coated surface. Data are expressed as fold change expression over control. *p < 0.05, **p < 0.01, versus vehicle.
cells treated with TROG also maintained their ability to polarize, which is consistent with the results from the static ICAM-1–binding assay and the flow cytometry data. Taken together, these results demonstrate that CLA prevents monocyte binding to ICAM-1 and reduces the capacity of these cells to polarize via a PPARγ-independent mechanism.

CLA reduces chemokine sensitivity in primary monocytes

To gain further insights into the effect of CLA on inside out activation of integrins, we examined the effect of CXCL12, which binds to the G protein–coupled receptor CXCR4, on CLA-treated monocytes. The signaling pathways initiated by CXCL12 and CXCR4 interactions induce a conformational change in β2 integrins, facilitating adhesion to ICAM-1 and subsequent polarization (28). To examine polarization, we pretreated human primary monocytes with CLA and subsequently exposed them to 100 ng/ml CXCL12. Shape change was monitored using F-actin staining. As with the previous experiments, cells treated with cis-9,trans-11 CLA or the CLA blend retained a rounded phenotype compared with all other treatments that induced polarization (Fig. 5A). Furthermore, we examined the mRNA expression of the CXCL12 receptor CXCR4. Both cis-9,trans-11 CLA and the CLA blend significantly inhibited CXCR4 expression by 1.88-fold (p > 0.05) and 1.49-fold (p > 0.01), respectively (Fig. 5B). TROG had no effect on its expression, indicating a likely PPARγ-independent mechanism through which CLA regulates CXCR4.

We extended these studies to establish whether CLA-treated monocytes failed to directionally migrate toward CXCL12 due to decreased CXCR4 expression. Using the THP-1 monocyte cell line, we tracked the movement of live cells toward 1 ng/ml CXCL12 over a 5-min period (Supplemental Fig. 3). We extended this to investigate the effect in HPBMCs. Plotting individual cell tracks over time revealed that cis-9,trans-11 CLA and the CLA blend inhibited both the distance traveled and the general directional movement toward CXCL12. These data demonstrate that cis-9,trans-11 CLA and the atheroprotective CLA blend reduce monocyte crawling and impair the ability of monocytes to migrate toward CXCL12 (Fig. 5C).

CLA-fed mice display reduced leukocyte adhesion to activated endothelium

To further elucidate whether CLA-mediated inhibition of monocyte adhesion and crawling plays a functional role in atheroprotection in vivo, we investigated the effect of CLA on monocyte function in the ApoE−/− model. We established a model of CLA-induced regression of pre-established atherosclerosis. IVM analysis of monocyte recruitment was performed in the femoral artery of ApoE−/− mice. ApoE−/− mice were fed ND (n = 12) or CD for 12 wk (n = 12) (Fig. 6A) and leukocyte velocity (v, μm/s) was quantified following injection of TNF-α (1 μg, 4 h) to induce endothelial cell activation, or saline control. CLA significantly inhibits leukocyte adhesion under basal and activated conditions compared with cholesterol-fed mice. CD-fed mice displayed decreased basal leukocyte rolling velocity compared with those fed ND. Mice supplemented with CLA had significantly faster rolling velocities compared with mice fed CD, making them more comparable with mice fed ND. (C) CD18 expression on monocyte subsets was determined by flow cytometry. Monocytes were separated based on their Gr1 and CD115 status, and the expression of CD18 was quantified. Circulating proinflammatory Gr1+/CD115+ monocytes in mice fed CD had a higher percentage of CD18 expression compared with mice fed ND. CLA supplementation significantly downregulated CD18 expression on the proinflammatory monocyte subset. **p < 0.01 versus CD.
pared with mice fed CD, indicating that there was an impairment in their ability to bind to activated endothelium (Fig. 6A). In the next series of experiments, we measured the rolling velocities of leukocytes in the femoral artery both in the presence and absence of TNF-α. In general, leukocytes from mice on CD displayed a significantly decreased basal rolling velocity compared with those fed ND (p < 0.01). Moreover, mice supplemented with CLA had significantly faster rolling velocities compared with those fed CD, making them more comparable to mice fed ND. However, treatment with TNF-α blunted the effect of CLA on leukocyte rolling velocity. This is not surprising, because rolling velocity is more dependent on expression of endothelial selectins and their corresponding ligands, rather than integrin expression, which, as identified through our in vitro experiments, is regulated by CLA. Finally, CLA had no effect on rolling leukocyte flux, suggesting that its effect is not due to a decreased number of leukocytes recruited. These data provide evidence that CLA inhibits leukocyte adhesion to the endothelium in vivo during regression of atherosclerosis.

**CLA preferentially downregulates CD18 expression on Gr1+/CD115+ monocytes**

Having shown that mice on a CLA-supplemented diet had impaired leukocyte adhesion compared with those fed CD, we aimed to identify whether there were changes in CD18 expression on monocyte subsets. Leukocytes were isolated from mice on the CD or CLA-supplemented diet and phenotyped using flow cytometry. Gr1+/CD115+ monocytes are described as inflammatory monocytes, whereas Gr1−/CD115+ monocytes are described as patrolling monocytes (32). Monocytes were separated based on their Gr1 and CD115 status, and the expression of CD18 was quantified. Circulating Gr1+/CD115+ monocytes in mice fed CD displayed a higher percentage of CD18 expression (37.7%) compared with mice fed ND (21.4%), although this difference was not significant. However, the percentage of CD18 expression on this monocyte subset in CLA-supplemented mice was significantly downregulated (4.2%, p = 0.012) compared with those fed CD (Fig. 6C). Interestingly, CD18 expression on Gr1+/CD115+ monocytes was not significantly different among the groups fed ND (42.8%), CD (41.1%), or CLA-supplemented diet (38.2%). Thus, CLA specifically targets CD18 expression on inflammatory Gr1+/CD115+ monocytes in vivo. Together, these data show that CLA mediates an effect on firm adhesion, cell polarization, and chemotactic migration of HPBMCs. (Fig. 7).

**Discussion**

CLA has the unique property of inducing regression of pre-established atherosclerosis in vivo (20, 31). The predominant bioactive CLA forms are the cis-9,trans-11 and trans-10,cis-12 isomers, and a 80:20 ratio blend approximates their relative levels during dietary intake (33). Our previous studies showed that a 80:20 cis-9,trans-11:trans-10,cis-12 CLA blend induces regression of pre-established atherosclerosis in the ApoE−/− murine model, despite continued high cholesterol challenge. Recently, we showed that the monocyte/macrophage cell is the cellular target through which CLA mediates this profound effect (31). Understanding the mechanism(s) through which CLA mediates monocyte function in the context of regression may facilitate the identification of novel mechanisms or pathways that limit pre-established disease.

In this study, we demonstrated that cis-9,trans-11 CLA and the atheroprotective CLA blend, but not trans-10,cis-12 CLA, inhibits the adhesion of monocytes to activated endothelial cells through the regulation of CD18 expression. For our experimental analysis we used 10 μM of CLA isomers in accordance with our previously published data and based on the results from the MTT assays.

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**FIGURE 7.** Effects of CLA on firm adhesion, cell polarization, and chemotactic migration of HPBMCs. CLA isomers and the 80:20 blend (cis-9,trans-11:trans-10,cis-12), acting via a PPARγ-dependent mechanism, decrease the expression of CD18 (β-chain) by β2 integrins, decreasing complex formation between α and β subunits in the Golgi apparatus and reducing the number of integrins expressed on the external surface. As a consequence, fewer LFA-1 and Mac-1 molecules adhere to endothelial ICAM-1. In addition, CLA acts via a PPARγ-independent manner on the adhesive/migratory CXCR4/CXCL12 pathway, significantly inhibiting CXCR4 expression and subsequently decreasing the interaction with CXCL12 cytokine, leading to a minor initiation of an “inside out” pathway. As result, partially, but incompletely, activated β2 integrins influence the interaction between CD18 of both LFA-1 and Mac-1, with actin filaments necessary for cell movement. In this way, cytoskeletal rearrangement of the monocyte is also inhibited, preventing monocyte adhesion, polarization, and, consequently, chemokine-targeted migration.
described above. Indeed, to the best of our knowledge, 10 μM is the lowest concentration of CLA isomers used in experimental analysis of HPBMCs. For example, Platt and El-Sohemy (34) treated primary human CD14+ monocytes with 50 μM of CLA isomers, whereas other investigators used 30 μM of those two CLA isomers to treat both human primary and THP-1 monocytes (35). The differential effects of the two most abundant CLA isomers on the inhibition of monocyte adhesion to activated endothelial cells are consistent with previous studies (36) that identified the divergent effects of the two CLA isomers in atherosclerosis. A recent study (37) demonstrated that cis-9, trans-11 CLA decreases Mac-1 expression on macrophages through the suppression of CD11b. However, that study did not investigate potential changes in CD18 expression. The biosynthesis of β2 integrins occurs with the assembly of the α- and β-chains in the endoplasmic reticulum, after which they are transported to the cell surface via the Golgi apparatus. In the absence of a pairing β-chain, the α-chain is not shuttled to the cell membrane (38). Our data are consistent with this paradigm of integrin assembly. We showed that although the mRNA and protein expression of both α-chains are unaffected, both are reduced on the cell surface as a consequence of CD18 inhibition (Fig. 7). It should be noted that HPBMCs were isolated using adhesion onto plastic, rather than a negative-selection method. Although our method ensures a pure population of monocytes, the method used may induce a state of pre-activation.

CLA was reported to mediate its effect, in part, via activation of PPARγ. However, our previous work showed that CLA inhibits monocyte migration via a PPARγ-dependent and -independent mechanism (21). In the current study, the PPARγ agonist TROG reduced CD18 mRNA and total protein expression. We confirmed that CLA mediates its effect on CD18 expression via a PPARγ-dependent mechanism because pretreatment with the PPARγ antagonist GW9662 abolished the effect of CLA on CD18 mRNA expression. Paradoxically, flow cytometry analysis showed that TROG increased the levels of surface CD18 expression. Although this may appear contradictory, our data are in keeping with previous studies. For example, Hirasawa et al. (39) demonstrated that TROG could reduce the binding of eosinophils to ICAM-1, but it did not downregulate the surface expression of CD11a, CD11b, or CD18, indicating that TROG may influence the activity of β2 via an alternative mechanism.

The binding of CXCL12 to the G protein receptor CXCR4 is an important mediator in the initial stages of leukocyte adhesion and extravasation to sites of inflammation (40). CXCL12 is present at high concentrations in atherosclerotic lesions where it is released by smooth muscle cells, endothelial cells, and macrophages (41). Engagement of CXCL12 with CXCR4 triggers a signaling pathway that induces integrins to switch from a low- to a high-affinity state. Hence, the activities of CXCR4 and β2 integrins are inextricably linked in the process of trafficking monocytes to sites of inflammation. We show that CLA inhibits CXCR4 expression and results in a failure of monocytes to migrate toward CXCL12. From our analysis, CLA-regulated expression of CXCR4 is not under the direct control of PPARγ, because the agonist TROG had no effect on its expression, highlighting the complexity of CLA signaling. However, although the data for CLA are consistent, in instances where CLA decreases adhesion to activated endothelial cells, reduces CXCR4 expression, and hence, chemotaxis to CXCL12 (Fig. 5), the data for TROG are conflicting. Although the PPARγ agonist TROG had no effect on CXCR4 expression, it inhibited chemotaxis to CXCL12. This may be due to the fact that TROG was shown to inhibit the phosphorylation of focal adhesion kinase (FAK), which is central in the induction of cell motility. FAK can mediate its effect downstream of integrin signaling to induce polymerization of actin. Indeed, it was shown that the PPARγ antagonist GW9662 has no effect on the ability of TROG to regulate FAK, highlighting that TROG itself can mediate PPARγ-independent effects in cells (42). Thus, it is feasible to suggest that the inability of TROG-treated monocytes to migrate toward CXCL12 is due to a general effect on cell motility and is independent of CXCR4. Further studies will be required to elucidate the complete mechanism behind CXCR4 regulation; however, CLA was shown to regulate other transcription factors beyond the PPARs, including ERK (43) and NFκB (44).

Murine monocytes can be classified into two main functional subtypes, inflammatory monocytes (Gr1+/CD11b+) and resident/patrolling monocytes (Gr1+/CD11b+), which have distinct properties (45). Resident monocytes patrol blood vessels in steady states but can also be recruited to inflammatory tissue (46) where they are thought to differentiate toward an M2 macrophage phenotype with wound healing and anti-inflammatory properties (7). Inflammatory monocytes are significantly increased during hypercholesterolemia and give rise to macrophages present in atherosclerotic plaques (47). Inflammatory monocytes preferentially differentiate toward M1 macrophages and TNF-α/IL-1β-producing dendritic cells (7). Although both M1 and M2 macrophages are present in plaques, they have different distribution patterns (48); however, a balance between the two subtypes is an important factor in plaque progression (49). We showed that CLA has the effect of downregulating CD18 on inflammatory monocytes in vivo and, thus, this may have a dramatic influence on the microenvironment within established plaques by shifting the balance toward the recruitment of resident monocytes.

To conclude, in this study we demonstrated that CD18 expression is downregulated by CLA, resulting in monocytes with a deficit in their ability to adhere to activated endothelium through ICAM-1. In addition, the observed increased leukocyte rolling velocity within CLA-fed mice indicates that leukocytes are undergoing weaker and more fleeting interactions with the vascular endothelium, translating to decreased opportunities for leukocytes to interact with endothelial integrins and convert to firmly adherent cells. Mice fed a CD supplemented with the 80:20 blend of CLA demonstrates that CLA has a greater influence on CD18 expression in Gr1+/CD11b+ inflammatory monocytes than other leukocyte subsets. Further studies will be required to determine the overall relationship between downregulating β2 integrins on one monocyte subset versus another, as well as how this alters the microenvironment within plaques, leading to regression.

Acknowledgments
We thank Prof. Carl G. Gahnberg for the generous gift of anti-CD18 Ab (R2E7B). We also thank Dr. Alfonso Blanco Fernández, Director of the Flow Cytometry Core Facility (Conway Institute, University College Dublin) for assistance with flow cytometry.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Data

Supplemental Figure Legends

Supplemental Fig 1. Effect of PPARγ antagonist, GW9662 on CLA regulation of CD18 expression.
HPBMCs were pre-treated with GW9662 (10μM) for 2 hours prior to the addition of c9,t11 CLA or CLA blend (10μM) for 18 hours. mRNA CD18 expression was performed by real time PCR analysis. Data are mean +/- SEM.

Supplemental Fig 2. Static adhesion to ICAM-1 in HPBMCs.
Poly-L-lysine (PLL) was used as a positive control for monocyte adhesion. 2% BSA was used as negative control for adhesion, in the presence or absence of 5μg/ml ICAM-1. All treatments were compared in resting vs EGTA/MgCl₂ activated-monocytes. Monocyte adherent cell number is expressed as percentage relative to vehicle control (mean % adhesion ±SEM. *p<0.05; **p<0.01 vs positive or negative controls). (A) Fluorescent microscopy (bars: 150 mm) imaging of adherent monocytes DAPI labelled is shown. 10X images are representative of three independent experiments. (B) Spectrophotometric quantification of monocyte adhesion. (C) Transmission light microscopy (bars: 35 mm) and fluorescent microscopy (bars: 30 mm) imaging of adherent monocytes DAPI (blue, for nuclei) and AlexaFluor 647-phalloidin (red, for F-actin) labelled is shown. 20X images are representative of three independent experiments. Micrographs and spectrophotometric quantification showed that ICAM-1 significantly increases adhesion, only upon monocyte activation.
Supplemental Fig. 3. CXCL12-induced monocyte activation dose-response.

Monocyte cells were unstimulated (neg ctr) or either activated with increasing concentration of CXCL12 and, as reported on the x axis, analysed (A) every minute, over a total time of 5 minutes for THP-1 monocytes or (B) every two minutes, over a time of 10 minutes for HPBMCs. Live cell images were captured by transmission light microscopy. For both experiments, cells displaying movement over time (crawling cells) were manually counted for each snapshot and the average of three independent experiments (in triplicates) is reported in the graphs.
Supplemental Fig. 2