Circulation

ORIGINAL RESEARCH ARTICLE

Oxidized Low-Density Lipoprotein Receptor in Lymphocytes Prevents Atherosclerosis and Predicts Subclinical Disease

BACKGROUND: Although the role of Th17 and regulatory T cells in the progression of atherosclerosis has been highlighted in recent years, their molecular mediators remain elusive. We aimed to evaluate the association between the CD69 receptor, a regulator of Th17/regulatory T cell immunity, and atherosclerosis development in animal models and in patients with subclinical disease.

METHODS: Low-density lipoprotein receptor–deficient chimeric mice expressing or not expressing CD69 on either myeloid or lymphoid cells were subjected to a high fat diet. In vitro functional assays with human T cells were performed to decipher the mechanism of the observed phenotypes. Expression of CD69 and NR4A nuclear receptors was evaluated by reverse transcription–polymerase chain reaction in 305 male participants of the PESA study (Progression of Early Subclinical Atherosclerosis) with extensive (n=128) or focal (n=55) subclinical atherosclerosis and without disease (n=122).

RESULTS: After a high fat diet, mice lacking CD69 on lymphoid cells developed large atheroma plaque along with an increased Th17/regulatory T cell ratio in blood. Oxidized low-density lipoprotein was shown to bind specifically and functionally to CD69 on human T lymphocytes, inhibiting the development of Th17 cells through the activation of NR4A nuclear receptors. Participants of the PESA study with evidence of subclinical atherosclerosis displayed a significant CD69 and NR4A1 mRNA downregulation in peripheral blood leukocytes compared with participants without disease. The expression of CD69 remained associated with the risk of subclinical atherosclerosis in an adjusted multivariable logistic regression model (odds ratio, 0.62; 95% CI, 0.40–0.94; *P*=0.006) after adjustment for traditional risk factors, the expression of NR4A1, the level of oxidized low-density lipoprotein, and the counts of different leucocyte subsets.

CONCLUSIONS: CD69 depletion from the lymphoid compartment promotes a Th17/regulatory T cell imbalance and exacerbates the development of atherosclerosis. CD69 binding to oxidized low-density lipoprotein on T cells induces the expression of anti-inflammatory transcription factors. Data from a cohort of the PESA study with subclinical atherosclerosis indicate that CD69 expression in PBLs inversely correlates with the presence of disease. The expression of CD69 remained an independent predictor of subclinical atherosclerosis after adjustment for traditional risk factors.

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Clinical Perspective

What Is New?

- This study identifies CD69 as an oxidized low-density lipoprotein receptor in T lymphocytes that contributes to the regulatory action of the adaptive immune system, preventing atherosclerosis development.
- CD69 controls the onset and progression of atherosclerosis, and its mRNA expression is an independent marker of early subclinical disease in humans.
- Data from a cohort of asymptomatic individuals indicate that CD69 expression in circulating T cells correlates inversely with the presence and extension of subclinical atherosclerosis.
- The expression of CD69 remained associated with the risk of subclinical atherosclerosis after adjustment for traditional cardiovascular risk factors.

What Are the Clinical Implications?

- Oxidized low-density lipoprotein binding to CD69 confers a regulatory phenotype to human and mouse T cells, dampening Th17 responses and ameliorating atherosclerosis.
- Expression of CD69 in circulating cells might serve as a new biomarker for the presence of subclinical atherosclerosis.

Although lipid deposition in the vessel wall is the hallmark of atherosclerosis, for many years, it has been recognized that inflammation also plays a crucial role in the genesis and progression of the disease and the appearance of clinical manifestations (infarction, stroke).[1] Recently, it has been demonstrated that the modulation of the inflammatory response is a valid target to reduce the risk of atherothrombotic events in high-risk patients.[2] The interplay between lipid metabolism and immune response is not completely understood. In the last decade, the contribution of lymphocytes to atherosclerosis clinical manifestations has been highlighted, and effector T-cell responses seem to be exacerbated in hyperlipidemia.[3,4]

In vivo models of atherosclerosis suggest that regulatory T (Treg) cells suppress inflammatory responses and attenuate atherosclerosis.[5] Experimental therapies aimed to increase Treg cell population under high fat diet (HFD) conditions have conclusively demonstrated an important role for this T-cell subset in atherosclerosis attenuation.[6,7] The role of Th17 lymphocytes is more controversial. Although genetic or pharmacological inhibition of interleukin (IL)-17 significantly ameliorates atherosclerosis,[8,9] concomitant increases in both IL-17 and IL-10 lead to smaller plaques.[10] Another study reports bigger plaques in *ApoE*$^{-/-}$*IL-17*$^{-/-}$ mice.[11] A Th17/Treg imbalance has been reported in patients with coronary artery atherosclerosis, with a significant increase in Th17 and a decrease in Treg cells.[12,13]

The early lymphocyte activation antigen CD69 regulates Th17 and Treg cell differentiation. CD69-deficient mice display enhanced Th17 differentiation and defective Treg cell function,[14–16] resulting in an inability to resolve inflammation or to maintain immune tolerance in diseases such as arthritis, asthma, contact dermatitis, or myocarditis.[17–19] However, no differences were observed in the atheroma plaque formation in CD69$^{-/-}$ ApoE$^{-/-}$ mice.[20]

A key event in the process of atheroma plaque formation is low-density lipoprotein (LDL) peroxidation, which generates highly inflammatory and immunogenic oxidized LDL (oxLDL).[21] However, oxLDL can also elicit anti-inflammatory responses by activating peroxisome proliferator-activated receptor-γ[22] and liver X receptor or by upregulating transcription factors with anti-inflammatory activity such as NR4A nuclear receptors.[23,24]

The main objectives of this work were to analyze the role of CD69 lymphocyte expression in atherosclerosis development, the immune mechanisms involved, and its relation with human disease. Using chimeric *ldlr*$^{-/-}$ mice subjected an HFD as atherosclerosis model, we show that CD69 deficiency specifically on lymphocytes leads to an altered Th17/Treg equilibrium and a consequent increase in atheroma plaque size during an HFD. In vitro assays in human T cells showed that the interaction between oxLDL and CD69 activates the expression of *NR4A* transcription factors, skewing T cells toward a regulatory phenotype and dampening Th17 and Th1 responses. Thus, we describe an unexpected regulatory mechanism of the adaptive immune system that delays atherosclerosis development in hyperlipidemic conditions. Remarkably, our data from participants in the PESA (Progression of Early Subclinical Atherosclerosis) cohort[25] were in agreement with these results in that downregulated CD69 expression in peripheral blood leukocytes (PBLs) is associated with subclinical atherosclerosis in an adjusted multivariable logistic regression model.

METHODS

The data, methods, and study material will be available to other researchers for purposes of reproducing the results or replicating the procedures by contacting the corresponding authors.

Study Design

To analyze the role of CD69 during atherosclerosis development, we used chimeras from ldlr$^{-/-}$ CD45.1^{+} mice (the line was obtained in house by crossing and subsequent selection by genotype of B6;129S7-*Ldlr*^{tm1Her}/J with B6.SJL-Ptprc^{a}Pepc^{b}/BoyCrl, from Jackson and Charles River, respectively) and CD69$^{-/-}$ or CD69$^{+/+}$ B6 double reporter (dRep) for Treg cells (FIR mice, Foxp3–monomeric red fluorescent protein [mRFP])

and Th17 cells (IL-17A–IRES–enhanced green fluorescent protein [eGFP]), hereafter *cd69*$^{-/-}$*dRep* and *cd69*$^{+/+}$*dRep*, respectively. dRep mice allow us to monitor the presence of live Treg cells and Th17 cells throughout the experiment. To evaluate the influence of immune cell CD69 expression during atherosclerosis development, Ldlr$^{-/-}$ mice were irradiated and reconstituted with bone marrow (BM) from *cd69*$^{-/-}$*dRep* mice or *cd69*$^{+/+}$*dRep*. Next, atherosclerosis development was evaluated in mice proficient or deficient for CD69 only in the myeloid compartment (MC; hereafter MC *cd69*$^{+/+}$ and MC *cd69*$^{-/-}$) or lymphoid compartment (LC; hereafter LC *cd69*$^{+/+}$ and LC *cd69*$^{-/-}$). For a detailed description of chimeric mice design, see the online-only Data Supplement. After 6 weeks of BM reconstitution, mice were placed on an HFD (SSNIFF, S9167-E010) for 10 to 16 weeks. Immune response was evaluated in peripheral blood, draining (para-aortic) lymph nodes (LNs), nondraining (inguinal, axillary, mesenteric) LNs, and spleen by flow cytometry and quantitative polymerase chain reaction (PCR). All animal procedures were approved by the ethics committee of the Comunidad Autónoma de Madrid and conducted in accordance with the institutional guidelines that comply with the European Institutes of Health directives (2010/63/EU of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes [*Official Journal of the European Union*, 2010:53:33–79]).

Quantification of CD69 and NR4A Gene Expression in the PESA Study

Expression of CD69 and nuclear receptors NR4A1 was evaluated by reverse transcription PCR with Taqman probes in PBLs of a subset of the participants from the PESA study.[25] This study prospectively enrolled 4184 asymptomatic participants 40 to 54 years of age to evaluate the systemic extent of atherosclerosis in the carotid, abdominal aortic, and iliofemoral territories by 2-dimensional/3-dimensional ultrasound and coronary artery calcification by computed tomography at baseline and 3 and 6 years after enrollment for follow-up studies. Participants were then defined as free of atherosclerosis (no disease; no presence of plaque and a coronary artery calcium score of zero) or with evidence of focal (1 site affected) or generalized (4–6 sites affected) subclinical atherosclerotic disease.[25] Following a general study strategy (ie, not for this specific analysis), 480 individuals of the whole cohort were retrospectively selected on the basis of the extent of subclinical atherosclerosis to perform molecular tests. Individuals were included in this subcohort, prioritizing those with more territories with evidence of plaque at baseline. For those presenting a tie, individuals with coronary artery calcification score ≥1 were included. Control subjects were then selected from those individuals without plaques and matched with the chosen cases on the basis of age, sex, family history of cardiovascular disease, dyslipidemia, and hypertension. From this subpopulation of 480 individuals, we selected 305 male participants classified as without disease (n=122) or with focal (n=55) or generalized (n=128) disease to test the expression of CD69 and NR4A1 by reverse transcription PCR. General characteristics of this specific subcohort are shown in Table I in the online-only Data Supplement.

Monitoring Th17 and Treg Cells in the In Vivo Model

To assess the immune response, the percentages of IL-17–eGFP^{+} or Foxp3-RFP^{+} cells in peripheral blood CD4^{+} T cells were monitored throughout the experiment by flow cytometry. The presence of Th17 and Treg cells was also evaluated at the end point of the experiment in aortic arch, spleen, nondraining (axillary) LNs, and draining (para-aortic) LNs.

OxLDL Binding Assays

Jurkat T (JK) cells or rat basophilic leukemia (RBL) cells stably transfected or not with CD69 were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate–labeled (Dil) LDL in their native and oxidized form in the presence or not of unlabeled lipoproteins. Lipoprotein binding was determined with flow cytometry. In some cases, blocking anti-CD69 antibody was added.

Human CD4^{+} T Cell Polarization

For human Th1, Th17, and Treg cell polarization, CD4^{+} T cells were purified from peripheral blood of healthy donors and incubated with a specific cocktail of recombinant cytokines. After the indicated days of culture, cells were analyzed in a FACSCanto Flow Cytometer. When indicated, oxLDL (50 μg/mL) and anti-CD69 (20 μg/mL) were added to the cultures.

Assessment of CD69 and NR4A Transcripts

Expression of mRNA levels of CD69 and NR4A nuclear receptors was analyzed in PBLs from patients with subclinical atherosclerosis and healthy subjects with reverse transcription PCR with Taqman probes (Applied Biosystems). Expression of NR4A receptors was also assessed in Jurkat T cells and human primary lymphocytes in the presence or not of oxLDL. When indicated, blocking anti-CD69 antibodies were also added. NR4A and CD69 mRNA expression was also determined in para-aortic LNs and peripheral blood lymphocytes from mice.

Tissue Processing and Immunohistochemistry

For plaque area assessment, 5-μm-thick sections at 100-μm intervals were collected starting at the origin of the aortic valve cusps. Sections were stained with Oil Red O staining, (Sigma-Aldrich) and hematoxylin, and lesion size was analyzed with ImageJ software. For Masson trichrome staining, 7-μm-thick sections at 100-μm intervals were collected. Sections were stained with the Masson-Goldner staining kit (Merck). For specific staining, anti-F480 antibody was purchased from Abcam (ab6640) and anti-CD3 from Santa Cruz Biotechnology (sc-1127). OxLDL was detected by immunofluorescence with rabbit anti-mouse oxLDL from Abcris.

Statistical Analysis

In vivo experiments were performed according to a randomized complete block design (treatments and different

time points have been taken into account) or a fully randomized design. To determine significant differences between 2 means, *P* values were calculated by unpaired *t* test or Mann-Whitney *U* test according to normality test, and differences among >2 means were analyzed by 1-way ANOVA or the Kruskal-Wallis test. To account for multiple comparisons, the Tukey or Bonferroni posttests were used to compare selected pairs of means and all pairs of means, respectively. When indicated, the Wilcoxon signed-rank test was used to analyze paired data. For differences in human T-cell differentiation assays, data were analyzed with the Friedman test and the Dunn multiple-comparison test. In kinetic experiments with the same mice, 2-way repeated-measures ANOVA with the Sidak multiple-comparison post hoc test was performed. Differences were considered significant at $P<0.05$.

Table I in the online-only Data Supplement shows demographics and cardiovascular risk factor statistics of the PESA subcohort selected for the quantitative PCR assay. Statistics were calculated with Stata (StataCorp, College Station, TX). Data are expressed as mean±SD, median and interquartile range, or number (percent). *P* values are derived from ANOVA for log-transformed continuous variables and χ^2 for categorical variables, except for those variables with n<6, for which the Fisher test was used.

The Cuzick extension of the Wilcoxon rank-sum test was used to assess the significance of the trend in the quantitative PCR data across the 3 ordinal groups allowing for ties. After that, 1-way ANOVA followed by the Tukey posttest was used to identify significant differences between each stage of subclinical atherosclerosis and baseline. Expression levels measured by quantitative PCR were normalized and considered in the log2 scale to fulfill the normality assumption. We then focused on the 2 most extreme groups (no disease versus generalized disease) and used a multivariable logistic regression model to assess the association of CD69 with subclinical atherosclerosis independently of known cardiovascular risk factors (age, smoking, family history of cardiovascular disease, dyslipidemia, hypertension, diabetes mellitus), the expression of NR4A1, oxLDL levels, and volume of different leukocyte subsets. These variables included in the model were found to be statistically significant with a value of $P<0.1$ in a univariable logistic regression model. Odds ratio, 95% CI, and *P* value are reported. Traditional cardiovascular risk factors were determined from blood samples and interviews as follows: (1) diabetes mellitus: fasting plasma glucose ≥126 mg/dL or treatment with insulin or oral hypoglycemic medication; (2) arterial hypertension: systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or use of antihypertensive medication; (3) hypercholesterolemia: total cholesterol ≥240 mg/dL, LDL cholesterol ≥160 mg/dL, high-density lipoprotein cholesterol <40 mg/dL, or use of lipid-lowering drugs; (4) smoking: current smoking status and a lifetime consumption of >100 cigarettes; and (5) family history of cardiovascular disease: first-degree relative diagnosed with atherosclerosis before 55 years of age in men and 65 years of age in women. Linear regression models were used to assess the correlation between the expression levels of CD69 with NR4A1 receptors. All these analyses were implemented with the R statistical software (www.r-project.org).

RESULTS

Lack of CD69 on Lymphocytes Exacerbates Atherosclerotic Plaque Formation

To evaluate the role of CD69 expression on BM-derived cells in the development of atherosclerosis, male Ldlr$^{-/-}$ mice were irradiated and reconstituted with BM from *cd69*$^{-/-}$ dRep for Foxp3-mRFP and IL-17A–eGFP[26] mice (BM *Cd69*$^{-/-}$) or WT-dRep littermates (BM *Cd69*$^{+/+}$; Figure IA in the online-only Data Supplement). From week 13 on, we found increased Th17/Treg cells ratio in PBLs of chimeric mice (Figure IB in the online-only Data Supplement), indicating a polarization toward proinflammatory phenotype in the absence of CD69. After 10 weeks of HFD, mice reconstituted with *cd69*$^{-/-}$*dRep* BM displayed a significantly high increase in IL-17–eGFP^{+} cells in para-aortic LNs, with higher absolute numbers compared with BM *Cd69*$^{+/+}$ mice (Figure IC and ID in the online-only Data Supplement). In agreement, the percentage but not cell numbers of Foxp3mRFP^{+} cells was significantly reduced in the absence of CD69 (Figure ID in the online-only Data Supplement), indicating that Treg cell recruitment to para-aortic LNs is not compromised. The percentage of CD4^{+} IL-17–eGFP^{+} cells was increased in the aortic arc of BM *Cd69*$^{-/-}$chimeras, whereas there is a tendency for decreased CD4^{+} and FoxP3^{+} cells (Figure IE and IF in the online-only Data Supplement). Collectively, flow cytometry data indicate that hyperlipidemia induces high proinflammatory activity and a disrupted Th17/Treg cells balance in the absence of CD69. Histochemical studies revealed more extensive lesions and necrotic cores in aortic valves from BM *Cd69*$^{-/-}$ chimeras compared with the BM *Cd69*$^{+/+}$ control group (Figure IG and IH in the online-only Data Supplement). Atheroma plaques in both groups consisted mainly of F4/80^{+} foam cells, although we found a higher infiltration of CD3^{+} lymphocytes into atheroma plaques in the BM *Cd69*$^{-/-}$ group (Figure II and IJ in the online-only Data Supplement). Peripheral blood leukocytes were analyzed during an HFD with no significant changes between BM *Cd69*$^{+/+}$ and BM *Cd69*$^{-/-}$ mice (Figure IIA and IIB in the online-only Data Supplement). Because CD69 has been also related to the maintenance of T-cell helper memory,[27] the CD44^{hi} CD62L^{lo} memory T-cell subset was also analyzed. Naïve T cells decrease with an HFD whereas memory T cells increase in both chimeric mice, suggesting that CD69 is not playing a role in the maintenance of T-helper memory cells in this model (Figure IIC in the online-only Data Supplement). Moreover, we found that the ratio between dendritic cells and Treg cells during HFD remains equal in both chimeric mice (Figure IID in the online-only Data Supplement). These data suggest that Th17 and Treg cell proportions are altered under the

course of HFD in BM Cd69$^{-/-}$ chimeric mice, whereas the other leukocyte subsets remained unaltered.

To address whether the observed phenotype in BM *Cd69*$^{-/-}$ mice is specific to the LC or MC, we generated mixed BM chimeras proficient or deficient for CD69 in either the LC (LC *Cd69*$^{+/+}$ and LC *Cd69*$^{-/-}$ groups) or MC (Figure 1A and Figure III in the online-only Data Supplement). The specific deletion of CD69 on lymphocytes and myeloid cells was confirmed by flow cytometry analysis in the blood of hyperlipidemic mice (Figure 1A). We observed a significantly enhanced plateau of Th17 response in the peripheral blood of the *LC Cd69*$^{-/-}$ group after HFD, whereas percentages of Foxp3 cells were significantly decreased compared with the *Cd69*$^{+/+}$ group (Figure 1B and 1C). On observation that peripheral Th17 responses started to diminish in the *LC Cd69*$^{-/-}$ group after week 16 on HFD (Figure 1C), mice were euthanized, and immune responses and plaque formation were assessed. IL-17–eGFP^{+} cells in para-aortic LNs from *LC Cd69*$^{-/-}$ mice were once again increased, whereas the percentages of Foxp3mRFP^{+} cells were comparable in the 2 groups (Figure 1D). However, the absolute number of Treg cells was significant decreased, with a significant increase in the number of Th17 cells (Figure 1D). Finally, atherosclerotic lesions were significantly more advanced with more extensive necrotic cores in the *LC Cd69*$^{-/-}$ group as assessed by Oil Red O and Masson trichrome staining (Figure 1E and 1F).

Myeloid cells are pivotal for atherosclerosis development.[28,29] We next performed mixed BM chimeras proficient or deficient for CD69 in the MC (MC *Cd69*$^{+/+}$ and MC *Cd69*$^{-/-}$; Figure IIIA in the online-only Data Supplement). We did not detect differences during HFD in Th17 or Treg cell dynamics in the periphery, at the site of inflammation (para-aortic LNs; Figure IIIB through IIID in the online-only Data Supplement), or in atheroma plaque formation (Figure IIIE and IIIF in the online-only Data Supplement). All the CD69-proficient and -deficient groups gained similar amounts of weight throughout the experiment. However, the circulating levels of lipids (free fatty acids, triglycerides, high-density lipoprotein, LDL, and cholesterol) were lower in LC *Cd69*$^{-/-}$ compared with LC *Cd69*$^{+/+}$, suggesting that the enhanced plaque formation in LC *Cd69*$^{-/-}$ was not attributable to a metabolic defect (Figure IVA through IVC in the online-only Data Supplement).

We conclude that specific CD69 deletion in the LC accounts for the increased proinflammatory phenotype and the enhanced atheroma plaque formation.

OxLDL Binds to CD69 on T Lymphocytes

The main receptor of oxLDL on vascular cells is lectin-like oxLDL receptor-1 (LOX-1), located in the same chromosomal locus immediately upstream to CD69. The LOX-1 and CD69 C-type lectin-like domains form very similar dimers (Figure 2A, left), unlike other C-type lectins such as dectin-1 or the macrophage mannose receptor (Figure V in the online-only Data Supplement). In LOX-1, the oxLDL-binding surface is located at the top of the dimer and contains a unique basic "spine" formed by the diagonal arrangement of arginine residues across the dimer surface (Figure 2A, top right).[30,31] Electrostatic surface representation of the CD69 C-type lectin-like domain dimer indicated that 4 arginine residues cluster at the center of the dimer and form a basic spine, similar to that of LOX-1 (Figure 2A, bottom right). Because this structural feature is proposed to be important for oxLDL recognition,[30] we hypothesized that CD69 binding to oxLDL particles could account for the phenotype observed in vivo.

Transfected Jurkat T cells stably expressing CD69 (JKCD69) on their surface bound oxLDL-DiI much more efficiently than untransfected JK (JKwt) cells (Figure 2B and 2C). JKCD69 cells bound oxLDL in a dose-dependent manner and showed a weaker binding of native LDL (Figure 2D). This binding was able to induce CD69 internalization in JKCD69 cells (Figure 2E). The specificity of oxLDL binding to CD69 was confirmed in blocking assays with anti-CD69 antibodies (Figure 2F).

CD69/oxLDL Binding Controls Th17/Treg Equilibrium and Expression of NR4A Nuclear Receptors

OxLDL exposure significantly reduced the mRNA levels of IL-8 and interferon-γ (IFN-γ) produced by JKCD69 cells but not in JKwt cells after activation (Figure 3A). Because of the role of CD69 in effector T-cell differentiation,[16] we evaluated human T-cell differentiation to effector phenotypes. Challenge of human CD4^{+} T cells with oxLDL diminished the percentage of IL-17^{+} and IFN-γ^{+} cells generated in response to Th17- or Th1-polarizing stimuli (Figure 3B and 3C) and favored Treg differentiation (Figure 3D). This effect was dependent on CD69, as demonstrated by the blockade with anti-CD69 antibodies (Figure 3B through 3D).

The NR4A orphan nuclear receptors have emerged as key regulators of the immune response, controlling the magnitude of the inflammatory processes; *NR4A1* and *NR4A3* are crucial for Treg cell development.[23,24] We assessed whether oxLDL regulates the expression of NR4A nuclear receptors (*NR4A1* and *NR4A3*) in human CD4^{+} T cells. As shown in Figure 3E, oxLDL enhanced *NR4A1* and *NR4A3* mRNA expression in T-cell receptor–activated human primary CD4^{+} T cells. An early induction of *NR4A3* was also evoked by oxLDL in JKCD69 cells but not in JKwt cells (Figure 3F), which was blocked by preincubation with anti-CD69 antibodies (Figure 3G) or shRNA, confirming the CD69-dependent effect of oxLDL (Figure 3H).

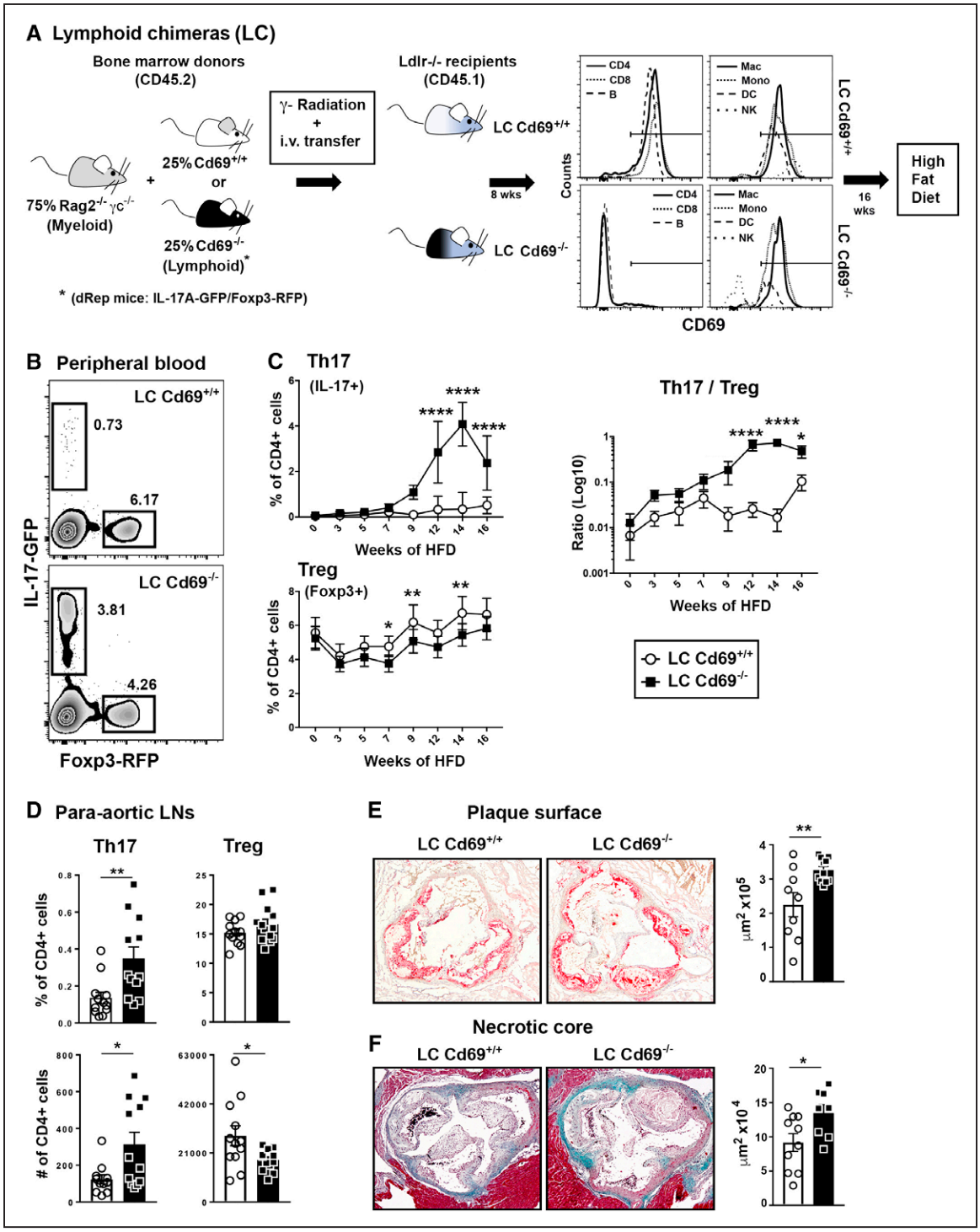

Figure 1. CD69 deficiency in lymphoid cells aggravates atherosclerosis.
A, Schematic illustrating the generation of lymphoid chimeras (LCs). Ldlr$^{-/-}$ (CD45.1^{+}) mice were lethally irradiated and reconstituted with mixed bone marrow (BM) from Rag2$^{-/-}$γc$^{-/-}$ plus BM from C57BL/6-Cd69$^{+/+}$ (LC *Cd69*$^{+/+}$) or C57BL/6-Cd69$^{-/-}$ (LC *Cd69*$^{-/-}$) (double-reporter [dRep]; interleukin [IL]-17–green fluorescent protein [GFP]$^{+}$/Foxp3–monomeric red fluorescent protein [RFP]$^{+}$) mice at a 3:1 ratio, respectively. Reconstitution of the lymphoid and myeloid compartments of CD45.2^{+} cells was assessed by fluorescence-activated cell sorting. Peripheral blood mononuclear cells (PBMCs) after 8 weeks of reconstitution were >90% CD45.2^{+}. Histograms show CD69 expression on CD4 and CD8 T cells, B cells (B), macrophages (Mac), monocytes (Mono), dendritic cells (DCs), and natural killer cells (NKs). PBMCs were stimulated overnight with anti–CD3/CD28 for lymphoid cells and lipopolysaccharide for myeloid cells of the indicated groups. **B**, Flow cytometry analysis of IL-17–GFP^{+} and Foxp3-RFP^{+} CD4 T cells in peripheral blood of LC CD69$^{+/+}$ and LC CD69$^{-/-}$ mice after 14 weeks of a high fat diet (HFD). **C**, Percentage of Th17 (IL-17–GFP^{+}), regulatory T (Treg; Foxp3-RFP^{+}) CD4^{+} T cells, and Th17/Treg ratio in peripheral blood leukocytes of LC *Cd69*$^{+/+}$ and LC *Cd69*$^{-/-}$ mice at the indicated time points after HFD initiation. n=15 mice per group (pooled from 3 independent experiments; error bars show SEM). *P* values were calculated by 2-way repeated-measures ANOVA (Sidak post hoc test). **P*<0.05. ***P*<0.01. **** *P*<0.0001. **D**, Percentage and absolute numbers of IL-17–GFP^{+}, Foxp3-RFP^{+} CD4 T cells in para-aortic lymph nodes (LN). n=12 mice per group. **E**. Oil Red O staining and quantification of plaque and necrotic core surface in aortic valves from LC *Cd69*$^{+/+}$ and LC *Cd69*$^{-/-}$ mice after 16 weeks of HFD. n= 8 mice/group. **F**, As in **E**, Masson trichrome staining and fibrosis quantification. n=7 mice per group Original magnification ×4. In **D** through **F**, error bars show SEM. **P*<0.05, ***P*<0.01 as determined by unpaired *t* test or Mann-Whitney *U* test.

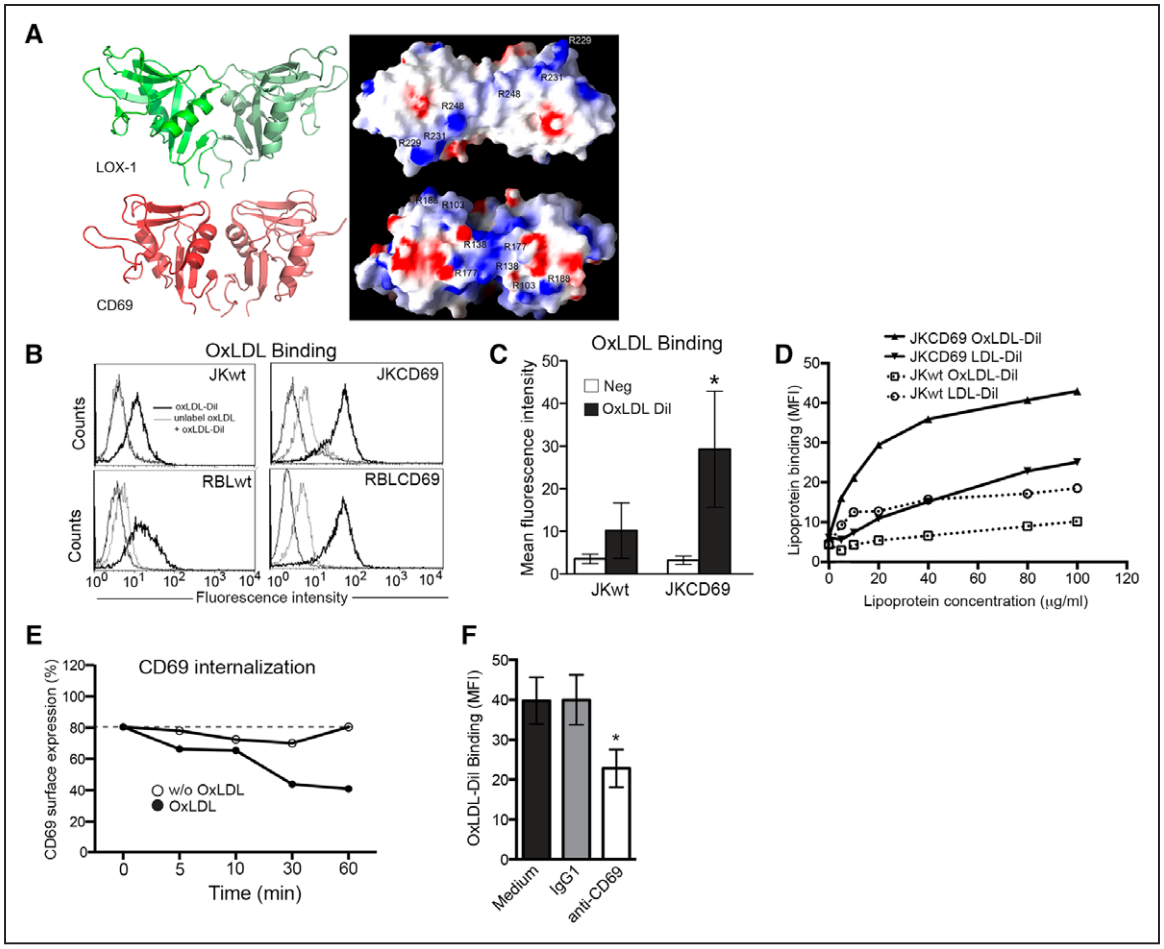

Figure 2. CD69 binding to oxidized low-density lipoprotein (oxLDL).
A, The LOX-1 and CD69 dimeric structures and their ligand-binding surfaces. Ribbon representations of the LOX-1 (Protein Data Bank code 1YXK) and CD69 (Protein Data Bank code 1E8i) C-type lectin-like domain (CTLD) structures (**left**; side views, prepared with PyMOL). Top views of the dimers with their surface charge distributions are shown on the **right** (prepared with GRASP). Arginine residues that form a basic "spine" across the dimer surface are labeled. **B**, Histograms show the binding of DiI-labeled oxLDL (solid line) to Jurkat T cells stably expressing CD69 (JKCD69) or rat basophilic leukemia cells (RBLCD69). Dotted line indicates the displacement of DiI-OxLDL by unlabeled lipoprotein. **C**, OxLDL binding to untransfected Jurkat T (JKwt) and JKCD69 cells (mean±SD from 10 independent experiments). *P<0.05 by Mann Whitney U test in both cell lines. **D**, Dose-response curves of oxLDL and native low-density lipoprotein (LDL) binding to JKwt and JKCD69 cells. **E**, CD69 internalization induced by oxLDL. JKCD69 cells were incubated with unlabeled oxLDL, and membrane-surface CD69 expression was analyzed by flow cytometry at the indicated times. Graph is representative of 1 of 3 experiments. **F**, Anti-CD69 antibodies inhibit the binding of oxLDL to JKCD69 cells (mean±SD from 4 experiments; *P<0.05). IgG1 indicates immunoglobulin G1; and MFI, mean fluorescence intensity.

In the in vivo model of atherosclerosis, the reporter GFP$^+$ Th17 cells and oxLDL localized closely at the atheroma plaque. Moreover, CD3$^+$ T cells and oxLDL colocalized at atheroma plaque (Figure VIA and VIB in the online-only Data Supplement). *NR4A1* and *NR4A3* mRNA expression was decreased in para-aortic LNs from BM *Cd69*$^{-/-}$ mice after 13 weeks of HFD (Figure 3I). Moreover, PBL expression of CD69 and *NR4A1* transcripts gradually declined in *ldlr*$^{-/-}$ mice during HFD administration (Figure 3J), indicating that these receptors are dynamically regulated in PBLs under these conditions.

CD69 Expression Is an Early Predictor of Subclinical Atherosclerosis

The PESA study is a prospective study that uses advanced imaging techniques to assess the presence of atheroma plaque in the main arteries of healthy individuals. Having observed a significant downregulation of CD69 as plaque formation progressed in mice, we compared CD69 and NR4A1 mRNA expression in PBLs from PESA participants with focal (1 affected site, n=55) or generalized (4–6 affected sites, n=128) subclinical atherosclerosis with that of PESA participants without any evidence of subclinical atherosclerosis (n=122; Table I in the online-only Data Supplement). The extent of subclinical atherosclerosis was assessed with advanced imaging techniques.[25] There is a gradient in CD69 expression across atherosclerosis extension stages; that is, CD69 expression decreases as disease progresses (trend test, P=0.006; Figure 4A). The same is true for NR4A1 (trend test, P=0.003; Figure 4A). These differences were particularly significant for the generalized subclinical disease group (Figure 4B and 4C). Thus, we focused on

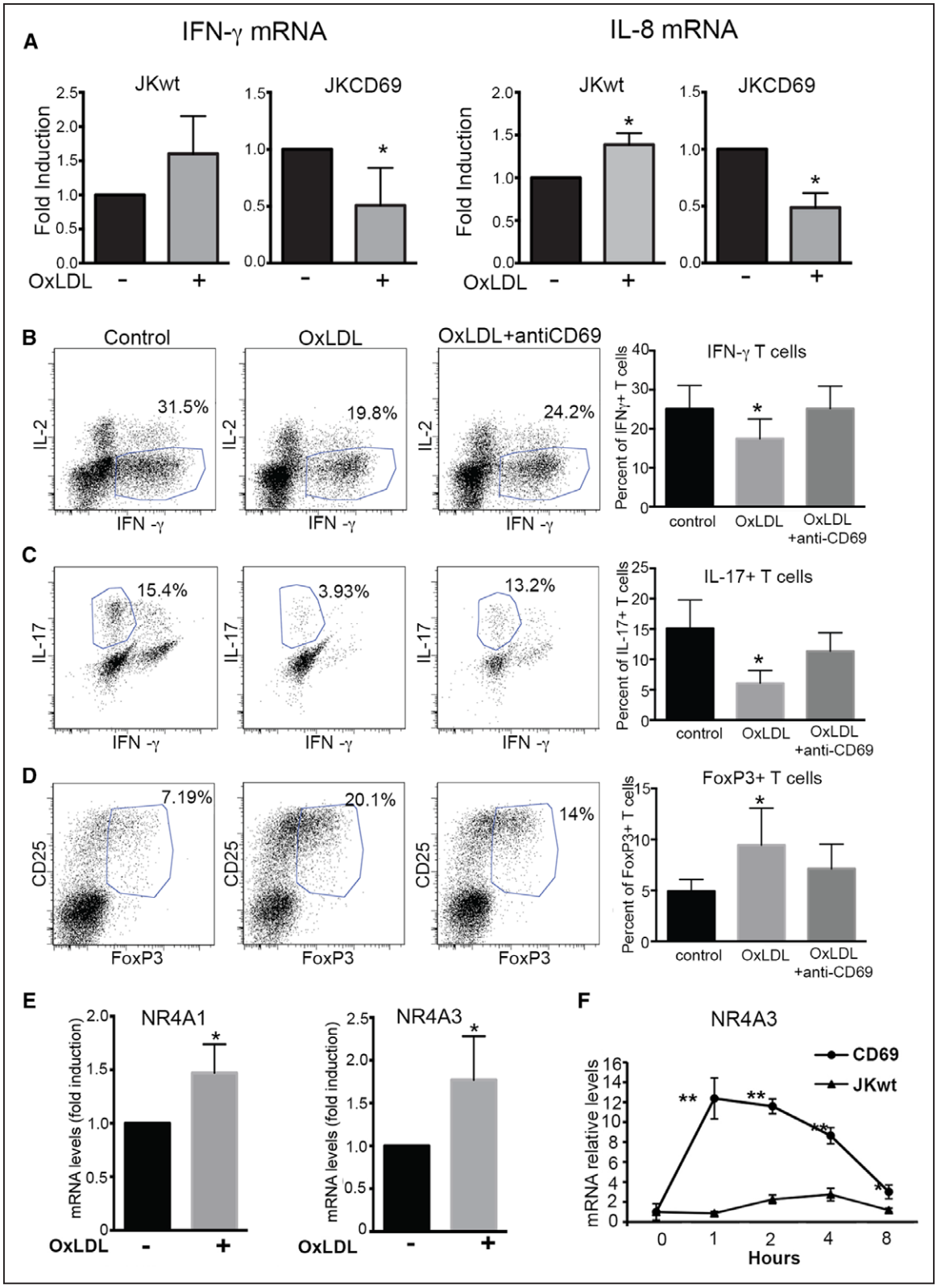

Figure 3. Oxidized low-density lipoprotein (OxLDL) binding to CD69 regulates the expression of NR4A receptors and cytokines in T cells.
A, OxLDL effect on phorbol 12-myristate 13-acetate/ionomycin-induced expression of interferon-γ(IFN-γ) and interleukin (IL)-8 in untransfected Jurkat T (JKwt) and Jurkat T cells stably expressing CD69 (JKCD69) cells. Data correspond to fold induction of mRNA levels (IFN-γ, n=6; IL-8, n=4) analyzed with the Wilcoxon signed-rank test. *P<0.05. **B** through **D**, Binding of oxLDL to CD69 blocks Th1 and Th17 differentiation and promotes regulatory T (Treg) cells. Human CD4+ T-cell differentiation was carried out in the absence (control) or presence of oxLDL (50 μg/mL) or oxLDL+anti-CD69. After corresponding days of culture, the percent of IFN-γ+ T cells (**B**), percent of IL-17+ T cells (**C**), and percent of CD25+Foxp3+ T cells (**D**) were determined by flow cytometry. Data correspond to 4 independent experiments, and bars represent mean±SEM of percent of positive cells. Data were analyzed with the Friedman test and Dunn posttest. *P<0.05 vs control. **E**, OxLDL induces NR4A1 and NR4A3 expression in activated human CD4+ T cells. Wilcoxon signed-rank test, *P<0.05 (n=4). **F**, Expression of NR4A3 in JKCD69 and JKwt cells treated (*Continued*)

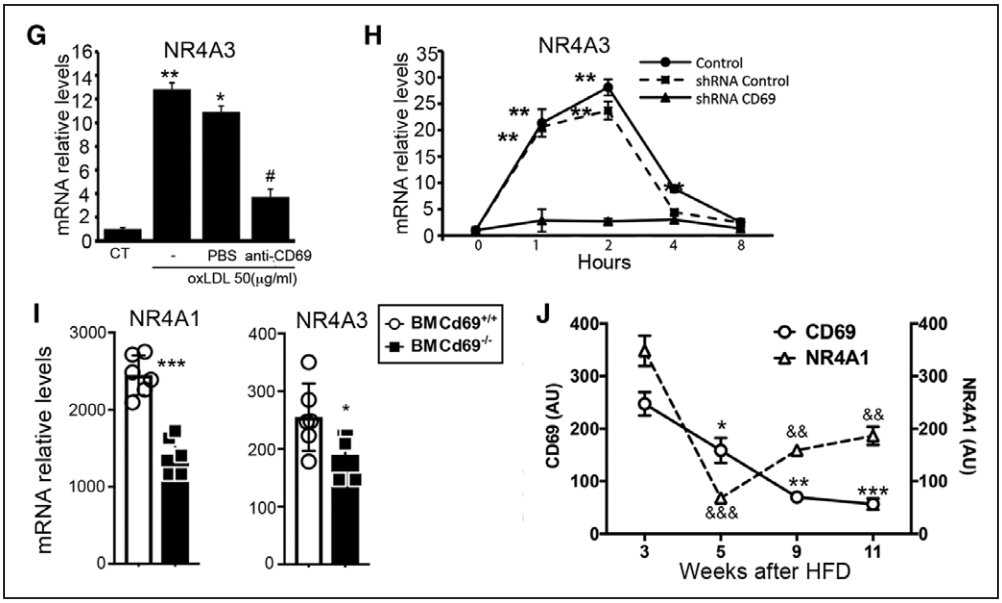

Figure 3 Continued. with oxLDL. Fold induction vs baseline (t=0). Data are mean±SD (n= 6) and were analyzed with 2-way ANOVA. ** *P*<0.0001, * *P*<0.01 vs JKwt at each time. **G**, Anti-CD69 inhibits oxLDL-induced NR4A3 expression in JKCD69 cells. Data are mean±SD (n= 6). Differences among groups were tested with the Kruskal-Wallis test. ***P*<0.001, **P*<0.01 vs control. #*P*<0.02 vs oxLDL. **H**, shRNA silencing of CD69 expression blocks oxLDL-induced NR4A3 expression in JKCD69 cells. Data are mean±SD (n= 6). Data were analyzed with 2-way ANOVA. ** *P*<0.0001 vs shRNA CD69. **I**, mRNA levels of the indicated nuclear receptors in para-aortic lymph nodes from total bone marrow (BM) chimeras at 13 weeks after a high fat diet (HFD). n=5 mice per group. Error bars show SEM. **P*<0.05, ****P*<0.001 as determined by unpaired *t* test. **J**, CD69 and NR4A1 mRNA relative expression in blood lymphocytes from *ldlr*$^{-/-}$ mice at different weeks after an HFD. Error bars show SEM. */&*P*<0.05, **/&&*P*<0.01, ***/&&&*P*<0.001 as determined by 1-way repeated-measures ANOVA. The Dunnett multiple-comparison test was performed between values at 5 to 11 weeks after an HFD vs 3 weeks after an HFD for CD69 (*) and NR4A1 (&) individually. AU indicates arbitrary units.

the control group (no disease) versus generalized disease group and examined whether CD69 can be used as independent marker for subclinical atherosclerosis. After a univariable logistic regression analysis (Table II in the online-only Data Supplement) of relevant variables (known cardiovascular risk factors: age, smoking, BMI, hypercholesterolemia, hypertension, diabetes mellitus, family history of cardiovascular disease; peripheral count of different subsets of leucocytes; expression of NR4A1 and the levels of oxLDL and C-reactive protein), a multivariable logistic regression analysis determined that CD69 expression remained an independent predictor of atherosclerosis at an early stage (odds ratio, 0.62; *P*=0.0056; Table). Furthermore, CD69 levels correlated significantly with NR4A1 levels (Figure 4D and Table III in the online-only Data Supplement), supporting the notion of a common regulation pathway.

DISCUSSION

Our findings indicate that the absence of CD69 in the LC results in larger atheroma plaque formation in *ldlr*$^{-/-}$ deficient mice subjected to an HFD. We identify the functional interaction between CD69 and oxLDL as the mechanism responsible for the observed phenotype. Our clinical data support this concept because CD69 expression in peripheral leucocytes of subjects with subclinical atherosclerosis is downregulated compared with individuals without atherosclerosis. This finding was in agreement with the experimental evidence showing a downregulation of CD69 expression on T lymphocytes in mice on exposure to HFD. Despite a suggested role for oxLDL in adaptive immune responses, a putative receptor on T lymphocytes has remained elusive.[32] Our study demonstrates that binding of oxLDL to CD69 in human T cells has a protective effect against the inflammatory response through the expression of NR4A nuclear receptors, downregulating proinflammatory cytokines and promoting Treg differentiation.

Although the classic view is that oxLDL induces the recruitment of inflammatory cells to the subendothelial space, cells and tissues also respond to oxLDL through the inhibition of proinflammatory signaling pathways.[33,34] The NR4A subfamily of human nuclear receptors (NR4A1 [Nur77], NR4A2 [Nurr1] and NR4A3 [NOR-1]) can be induced in endothelial and smooth muscle cells by a range of stimuli (including oxLDL), regulating the expression of different molecules involved in the immune response.[35] NR4A overexpression decreases the levels of IL-1β, IL-8, and monocyte chemoattractant protein-1 inflammatory cytokines. Furthermore, NR4A1 and NR4A3 have been implicated in Treg differentiation.[23,24] Our data show that binding of oxLDL to CD69 in human T cells induces the expression of NR4A receptors. Moreover, the absence of CD69 during atherosclerosis development results in a lower expression of NR4A1 and NR4A3 in both PBLs and para-aortic LNs. The observation of the regulatory effect of CD69/oxLDL interaction in human Treg differentiation, together with the loss of Treg responses

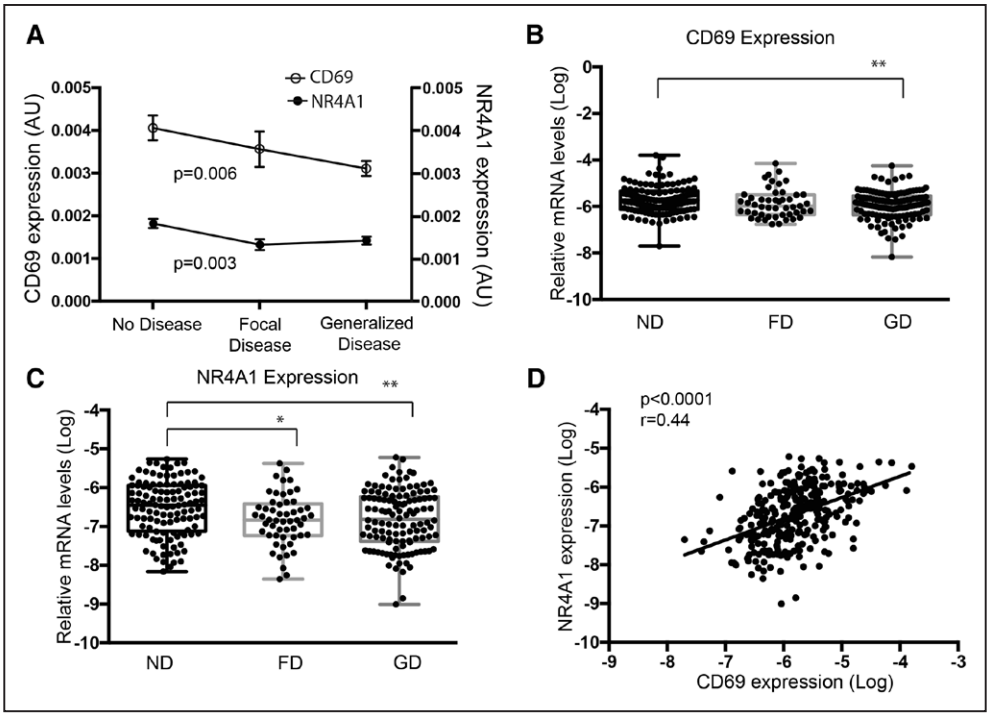

Figure 4. Individuals with subclinical atherosclerosis express low levels of CD69 and NR4A1.
A, mRNA expression of CD69 and NR4A1 is presented as mean±SEM in linear scale. Significance of the trend across groups was calculated with the Cuzick extension of the Wilcoxon rank-sum test to assess the significance of the trend across the 3 ordinal groups allowing for ties. **B**, mRNA levels of CD69 in peripheral blood leukocytes from healthy subjects (no disease [ND], n=122) and individuals with subclinical atherosclerosis (focal disease [FD], n=55; generalized disease [GD], n=128). **C**, mRNA levels of NR4A1 as in **B**. Data are expressed in log2 scale and were analyzed with 1-way ANOVA followed by Tukey posttests. * *P*<0.05. ***P*<0.005. **D**, Correlation between the expression of CD69 and NR4A1. The Pearson correlation test was used.

observed in lymphocyte CD69-deficient mice during atherosclerosis development, suggests that the modulation of NR4A nuclear receptors could participate in CD69 signaling/Treg differentiation. The more extensive atherosclerosis developed in the absence of CD69 could be associated, at least in part, with defects in Treg cell differentiation. Treg cells exert an atheroprotective function through the suppression of T-cell proliferation and secretion of anti-inflammatory cytokines and are protective during the initial phases of atherosclerosis not only by reducing atherosclerotic plaque formation but also by improving stabilization of the atherosclerotic lesions.[36–38]

Besides promoting Treg cell function, CD69 controls Th17 differentiation through the association of its cytoplasmic tail with the Jak3/Stat5 signaling pathway, regulating RAR-related orphan receptor-γ transcription and differentiation toward the Th17 lineage.[15] Recent evidence for the role of IL-17 in atherosclerosis has shed little light on the subject in both proatherogenic and antiatherogenic roles.[39,40] Under HFD, *ldlr*$^{-/-}$ mice deficient for CD69 in the LC developed exacerbated Th17 responses and more severe atherosclerotic lesions, supporting the role of IL-17 as a proatherogenic molecule. Despite previous reports supporting that IL-17 can either stabilize the plaque through collagen production or enhance the recruitment of proatherogenic cells through CXCL1 upregulation,[11,40] the former seems not to be the case in our model. Increased amounts of IL-17 seem to stabilize plaque formation when levels of IL-10 are also higher, namely in the presence of a proper

Table. Multivariable Logistic Regression Comparing Individuals With Generalized Subclinical Atherosclerosis and Individuals With No Disease

	Odds Ratio (95% CI)	*P* Value
log2(CD69)	0.62 (0.40–0.94)	0.0056
log2(NR4A1)	1.12 (0.80–1.58)	0.0441
Age	1.21 (1.11–1.33)	0.0000
Familial hypercholesterolemia	2.06 (0.90–4.87)	0.0630
Hypercholesterolemia	1.13 (0.58–2.22)	0.1168
Smoking	2.16 (1.00–4.87)	0.0027
Monocytes	0.06 (0.00–1.61)	0.4733
Lymphocytes	1.57 (0.66 –3.82)	0.0025
Leukocytes	1.61 (1.11–2.41)	0.0079
Diabetes mellitus*	Not applicable	0.0246
Oxidized low-density lipoprotein	1.02 (1.00–1.04)	0.0457

All covariates with a value of *P*<0.1 in the univariable analysis (Table II in the online-only Data Supplement) were considered.

**P* values were calculated with the likelihood ratio test to accommodate groups with 0 counts (diabetes mellitus), for which odds ratio estimates could not be calculated. Odds ratio and CIs were estimated with a generalized linear model.

regulatory response.[39] In our model, however, we have a concomitant increase of IL-17 and defective Treg development and function.

Previous results indicated that the regulatory action of CD69 during atherosclerosis was lost in *ApoE*$^{-/-}$ mice because, compared with the double-knockout group (*Cd69*$^{-/-}$*ApoE*$^{-/-}$), no significant difference was observed in plaque formation.[20] LDLr$^{-/-}$ and ApoE$^{-/-}$ mice have been extensively used to study the mechanisms of atherosclerosis development but feature important differences, for example, in plasma proteins. The major accumulating lipoprotein in the plasma of LDLr$^{-/-}$ mice fed a high-cholesterol diet is LDL. Conversely, ApoE$^{-/-}$ mice accumulate cholesterol mostly in the very-low-density lipoprotein and chylomicron fractions.[41,42] This is a very important issue to be considered that could account for the differences observed between CD69$^{-/-}$/apoE$^{-/-}$ and CD69$^{-/-}$/Ldlr$^{-/-}$ models. Our data show that oxLDL binding to CD69 exerts an immune-regulatory function during atherosclerosis development. In the ApoE knockout model, the levels of LDL may not have reached the threshold that is required for signaling through CD69. An additional difference that should be taken into consideration is that Ldlr$^{-/-}$ lesions have higher T-cell density than the ApoE model,[43] meaning that Ldlr$^{-/-}$ mice have more T cells that could express CD69 to exert their function. Finally, ApoE$^{-/-}$ mice on an HFD develop plaques more rapidly and exhibit larger aortic lesions with larger necrotic cores than Ldldr$^{-/-}$ mice[41,42]; therefore, this high-intensity model could be masking the differences between the CD69-expressing and CD69-deficient animals. We found a significant decrease in the blood lipid profile of free fatty acids, triglycerides, high-density lipoprotein, LDL, and cholesterol in the *Cd69*$^{-/-}$ lymphoid chimeras. This "dissociation" of the immune profile of the organism from the metabolic parameters is quite intriguing; it seems to occur via mechanisms related mainly to the adaptive immune responses (in this case, lack of CD69 and Th17 propensity). Our data pave the way for further research on mechanisms that could contribute to atherogenesis.

Our results describe for the first time an oxLDL receptor on lymphocytes with an important function in the regulation of the adaptive immune response and atheroma plaque formation during an HFD. The chimeric *ldlr*$^{-/-}$ mouse models used shed light on the role of CD69/oxLDL functional interaction in lymphocytes and on the maintenance of immune homeostasis to protect medium and large arteries from severe atheroma plaque formation over time. Further studies of the new regulatory oxLDL/CD69 pair in human lymphoid cells during atherosclerotic disease progression will provide novel insight into targeting these pathways for the prognosis/treatment of cardiovascular diseases.

Recent data emphasized the link between the inflammatory response and atherosclerotic risk in the clinical arena.[2] However, the complex interplay between lipid metabolism and immune responses remains to be fully disclosed. Collectively, our in vivo data strongly indicate an important role for CD69 expression on lymphocytes during atherosclerosis development. To validate this new paradigm in humans, we assessed the expression of CD69 and NR4A in PBLs from a cohort of subjects with thoroughly characterized subclinical atherosclerosis. It is important to point out that all PESA participants included in the study were asymptomatic and free of events. The profile of CD69 expression detected in human samples was very similar to that observed in the in vivo model. If we consider focal disease and generalized disease as different stages with the same pathology, our data indicate that expression of CD69 gradually declines as disease progresses. Hence, we focused on the generalized disease group, which is still preclinical, to assess the association of the expression of CD69 with subclinical atherosclerosis, accounting for the effect of traditional risk factors, peripheral counts of different subsets of leucocytes, expression of NR4A1, and levels of oxLDL. The proof of clinical study more closely resembling the animal experiments was to compare subjects free of disease or no disease versus those with established but yet subclinical atherosclerosis or generalized subclinical disease. CD69 remained significantly associated with the extent of the disease after adjustment for risk factors. This finding underscores the putative role of CD69 as a potential marker for the detection of atherosclerosis at a preclinical stage.

The fact that atherosclerosis presence is well identified in a context different from acute conditions (eg, myocardial infarction) supports the causative role of CD69 expression on atherosclerosis development rather than a consequence of an acute event.

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Disclosures

None.

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